

## PHD

### The distribution and functions of melanin-concentrating hormone in lower vertebrates

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# THE DISTRIBUTION AND FUNCTIONS OF MELANIN-CONCENTRATING HORMONE IN LOWER VERTEBRATES

Submitted by Karen Francis  
for the degree of PhD  
of the University of Bath

1996

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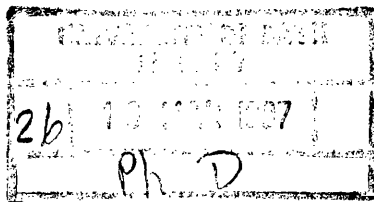
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## SUMMARY

This thesis describes studies into the distribution and functions of melanin-concentrating hormone (MCH) in fish and amphibia.

In the amphibian *Rana temporaria*, irMCH neurons are located in the dorsal and ventral infundibular nuclei (NID & NIV) of the posterior hypothalamus at all ages after stage TK XXII of development, but additional MCH groups appear in the pre-optic area and telencephalic lateral septal nuclei in gravid females, suggesting reproductive functions.

Immunoreactive MCH neurons in the NID/NIV first appear in the *Rana* tadpole during mid-metamorphic climax. Immunocytochemical and morphometric data indicate an increased activity at the time of emergence onto land. These neurons are similarly activated in adult frogs after 5 d exposure to 35% salt water. The potential involvement of MCH with osmoregulation is considered.

In trout, MCH neurons were also responsive to salinity. *In-situ* hybridization showed enhanced MCHmRNA in the *nucleus lateralis tuberis* (NLT) after 24 h exposure to 80% seawater. However, 100% seawater rapidly but transiently depressed MCH gene expression in the both the NLT and in the neurons above the lateral ventricular recess (LVR-MCH cells), activity returning to normal after 6 d despite persistent high plasma cortisol and osmotic pressure. The relative importance of plasma cortisol, osmotic pressure and stress in regulating MCH gene activity is discussed.

Immunoreactive MCH was also observed in mast-like cells in the *lamina propria* of the trout gut, as in the rat. The multiple factors affecting MCH and its potential functions in stress, osmoregulation, reproduction and immunity are discussed.

## CHAPTER ONE

### INTRODUCTION

1.1	General introduction	1
1.2	MCH peptide and precursor structures in fish and mammals	3
1.3	Colocalisation of MCH, $\alpha$ -MSH, hGRF & CRF brain immunoreactivity	5
1.4	MCH receptors	6
1.5	MCH distribution and colour change function in teleost fish	7
1.6	MCH as a modulator of stress in teleosts	9
1.7	MCH as a modulator of stress in rats	11
1.8	MCH distribution and antagonism with $\alpha$ -MSH in rat brain	13
1.9	The effects of osmotic stimuli on MCH in rat	15
1.10	MCH and feeding behaviour in rodents	16
1.11	MCH in humans	16
1.12	Aim of the present study	17

## CHAPTER TWO

### MATERIALS AND METHODS

2.1	Animal husbandry	20
2.1.1	Trout	20
2.1.2	Frogs	20
2.1.3	Toads	21
2.2	Collection of trout blood and plasma extraction	21
2.3	Collection of brain tissue	21
2.3.1	Trout	21
2.3.2	Amphibia	22
2.4	Immunocytochemistry	22
2.4.1	Wax embedded sections	22
2.4.2	Vibratome sections	23
2.5	Cortisol radioimmunoassay	24
2.5.1	Preparation of steroid	24
2.5.2	Separation of bound and unbound fractions	24
2.5.3	Cortisol standards	25
2.5.4	Tritiated cortisol and cortisol antibody	25
2.6	<i>In-situ</i> hybridization	25
2.6.1	Preparation of slides	25
2.6.2	3' end-labelling of oligodeoxynucleotide probe (MCH2)	26
2.6.3	Purification of the labelled probe using a QIAquick kit	27
2.6.5	Post hybridization washing	28
2.6.6	Autoradiography	28
2.6.7	Dipping slides in photographic emulsion	29

## CHAPTER 3

### DEVELOPMENTAL CHANGES IN MELANIN-CONCENTRATING HORMONE IN THE GRASS FROG, *Rana temporaria*.

3.1	Introduction	30
3.2	Materials and Methods	31
3.2.1	Amphibians	31
3.2.2	Preparation of brain tissue and immunocytochemistry	32
3.3	Results	33
3.3.1	MCH distribution in metamorphic stages of <i>Rana temporaria</i>	33
3.2.2	Comparison of MCH neuronal activity in pre- and post-emergent stages	33
3.2.3	Comparison with <i>Xenopus laevis</i> .	35
3.3	Discussion	36

## CHAPTER 4

### MCH EXPRESSION IN THE ADULT AMPHIBIAN

4.1	Introduction	44
4.2	Materials and Methods	46
4.2.1	Amphibians	46
4.2.2	Expt. 1.35% salinity exposure for 10d in <i>Xenopus laevis</i>	46
4.2.3	Expt. 2.35% salinity exposure for 5d in <i>Rana temporaria</i>	47
4.2.4	Expt. 3.35% salinity exposure for 5d in male <i>R. temporaria</i>	48
4.2.5	Expt. 4.Mapping MCH neurons and fibres in adult <i>R. temporaria</i>	48
4.2.6	Expt. 5.Investigating irMCH distribution in the reproductively mature <i>R. temporaria</i> using vibratome sections	49
4.3	Results	49
4.3.1	The effect of exposure to 35% salinity in <i>Xenopus laevis</i>	49
4.3.2	The effect of exposure to 35% salinity in <i>Rana temporaria</i>	52
4.3.3	Mapping MCH cell bodies and neuronal tracts in adult <i>R. temporaria</i> using wax embedded sections	57
4.3.4	Investigating irMCH distribution in the reproductively mature <i>R. temporaria</i> using vibratome sections	60
4.4	Discussion	64

## CHAPTER 5

### THE EFFECT OF SALINITY ON HYPOTHALAMIC MCH IN THE RAINBOW TROUT

5.1	Introduction	75
5.2	Materials and methods	77
5.2.1	Experiment 1.	77
5.2.2	Experiment 2	79
5.3	Results	79
5.4	Discussion	85



## CHAPTER 6

### AN ANALYSIS OF THE *IN-SITU* HYBRIDIZATION METHOD

6.1	Introduction	91
6.2	Method	91
6.3	Results and discussion	94

## CHAPTER 7

### MCH IN THE TROUT GUT

7.1	Introduction	103
7.2	Materials and methods	104
	7.2.1 Wax-embedded sections	105
	7.2.2 Frozen sections	105
7.3	Results	106
7.4	Discussion	108

## CHAPTER 8

GENERAL DISCUSSION	112
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APPENDIX	121
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REFERENCES	122
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## CHAPTER ONE

### INTRODUCTION

#### 1.1 General introduction

Nearly thirty years after its existence was first proposed (Enami, 1955) a melanophore-contracting hormone, or melanin-concentrating hormone (MCH) as it became, was purified from salmon pituitaries, and sequenced (Kawauchi *et al.*, 1983). Thought initially to be just a teleost hormone, responsible for causing skin pallor, further evidence of its existence in the brains of *Rana temporaria*, *Xenopus laevis* and the rat (Baker and Rance, 1983) subsequently prompted a wider interest in this little known peptide. After antisera against salmonid MCH (sMCH) were raised (Okamoto *et al.*, 1983; Wilkes *et al.*, 1984) the peptide was mapped in various vertebrate brains including teleost (Naito *et al.*, 1985; Batten and Baker, 1988; Bird *et al.*, 1989) and elasmobranch fish (Vallarino *et al.*, 1989), an amphibian (Andersen *et al.*, 1986), reptiles (Cardot *et al.*, 1994) and the rat (Skofitsch *et al.*, 1985). In invertebrates MCH immunoreactivity is present in the optic lobes of the locust and in the *pars intercebralis* of the locust and fleshfly (Schoofs *et al.*, 1988).

In vertebrates, MCH neurons are generally located within the hypothalamus and the neuronal tracts extend to various regions of the brain. In the case of teleosts, many MCH axons project to the pituitary gland (Naito *et al.*, 1985; Batten and Baker, 1988; Bird *et al.*, 1989) and in tilapia, MCH cell bodies even migrate into the pituitary stalk and neurohypophysis (Gröneveld *et al.*, 1995a). In other classes of vertebrates, however, relatively few MCH fibres extend to the neural lobe.

## Chapter one

The sequencing of sMCH, and later rat MCH (rMCH), permitted the elucidation of the cDNA sequences that encode the MCH preprohormone (ppMCH) in salmonids (Ono *et al.*, 1988; Minth *et al.*, 1989; Baker *et al.*, 1995) tilapia (Gröneveld *et al.*, 1993) mouse (Breton *et al.*, 1993a) rat (Nahon *et al.*, 1989; Thompson and Watson, 1990) and human (Presse *et al.*, 1990; Breton *et al.*, 1993b). In all cases, the MCH sequence is located at the C-terminus of the prohormone. In addition, determination of potential cleavage sites at dibasic residues within ppMCH has led to the recognition of other putative peptides that could be cleaved from the precursor. In mammals there are two such peptides, termed neuropeptide glutamic acid-isoleucine (NEI) and neuropeptide glycine-glutamic acid (NGE). In salmonids, the NEI equivalent is termed neuropeptide glutamic acid-valine (NEV) (Nahon *et al.*, 1991) or MCH gene related peptide (Mgrp) (Bird *et al.*, 1989; Gröneveld *et al.*, 1995c).

The role of MCH as a neurohypophysial colour change hormone appears to be restricted to the teleost fish. In other species the brain peptide is believed to act primarily as a central nervous system neuromodulator since very little MCH is found in the pituitary neural lobe and no measurable levels of MCH have been detected in rat or human plasma (Takahashi, *et al.*, 1995). As discussed later, it has been associated with the modulation of functions such as stress, appetite and various behaviours. Recently, however, MCH mRNA has been detected in peripheral tissues such as the stomach, testis and intestine of the rat (Hervieu and Nahon, 1995) and heart, intestine, spleen and testis of the mouse (Breton *et al.*, 1993a) although in amounts significantly lower than those found in the central nervous system.

## 1.2 MCH peptide and precursor structures in fish and mammals

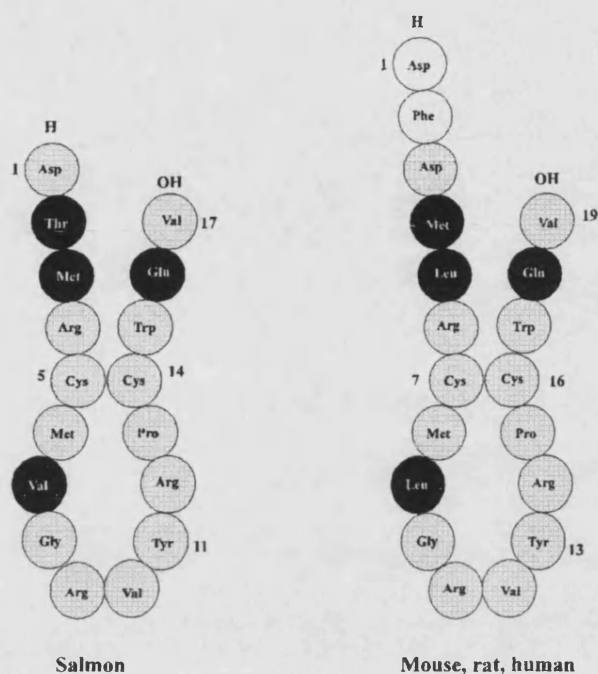


Fig 1 Representation of salmon and mammalian MCH. Identical residues are lightly shaded and substitutions are highlighted.

Salmonid MCH (sMCH) is a cyclic heptadecapeptide (Figure 1), the ring formed by a disulphide bridge between two cysteine residues, Cys<sup>5</sup> and Cys<sup>14</sup> (Kawauchi *et al.*, 1983). The ring structure is essential for function and any modification reduces MCH activity. The exocyclic arms may also affect the molecular shape and hence influence the potency of the molecule (Kawazoe *et al.*, 1987; Lebl *et al.*, 1989; Baker, 1991). Although sMCH is a unique peptide it bears a slight similarity to the C-terminal sequence of salmonid but not mammalian prolactin (Kawauchi *et al.*, 1983). Human, mouse and rat MCH are identical nonadecapeptides (Figure 1), differing from sMCH by a two amino acid extension on the N-terminal exocyclic arm and four substitutions (Vaughan *et al.*, 1989; Presse *et al.*, 1990;

Breton *et al.*, 1993a). Hence, excluding the two additional residues, there is 76% amino acid identity between mammalian and sMCH.

The sMCH precursor molecule, of 132 amino acids, is highly homologous between rainbow trout and chinook, coho and chum salmon, with 96% amino acid identity (Ono *et al.*, 1988; Minth *et al.*, 1989; Nahon *et al.*, 1991; Baker *et al.*, 1995). Since salmonids are tetraploid, they have two copies of the MCH gene, which share 86% identity (Baker, 1991). In the rainbow trout only one of these genes (MCH2) has been found to be expressed in the brain (Baker *et al.*, 1995) and the same may be true for coho salmon (Nahon *et al.*, 1991). The mammalian MCH peptide lies at the C-terminal end of a precursor of 165 residues which, apart from mature MCH peptide, shares little homology with the salmonid equivalent. Both salmonid and mammalian MCH precursors contain potential cleavage sites which enclose other peptides (Figure 2), and in magnocellular neurons the rat prohormone is known to be colocalised with a convertase enzyme, PC2, which cleaves the molecule at selective dibasic residues (Seidah *et al.*, 1993).

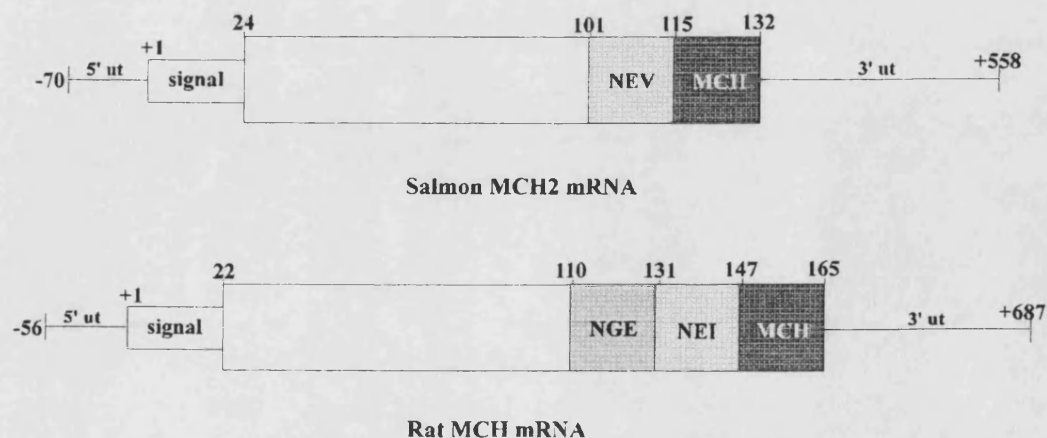


Fig 2 Structure of salmon MCH2 and rat MCH genes. ut = untranslated regions

## Chapter one

In rat ppMCH, cleavage between Lys<sup>129</sup>-Arg<sup>130</sup> and Arg<sup>145</sup>-Arg<sup>146</sup> liberates NEI which, having a glycine residue at the C-terminal, may be amidated (Nahon *et al.*, 1989). There is evidence that this form of NEI is actually processed from ppMCH and its release from cultured rat hypothalami has been demonstrated (Parkes and Vale, 1993). Cleavage between Lys<sup>109</sup> and Lys<sup>129</sup>-Arg<sup>130</sup> could release a second peptide, NGE, which has yet to be identified biochemically but whose immunoreactivity has been demonstrated in secretory granules (Fellmann *et al.*, 1993).

### 1.3 Colocalisation of MCH, $\alpha$ -MSH, hGRF and CRF brain immunoreactivity.

Sequencing of the other peptides within ppMCH led to an understanding of the observation by many groups that in certain hypothalamic neurons, MCH appears to be colocalised with  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) but not with other pro-opiomelanocortin (POMC) precursors such as adrenocorticotropin hormone (ACTH) or  $\beta$ -endorphin. This colocalisation is apparent in the brains of rat (Fellmann *et al.*, 1986; Naito *et al.*, 1986), carp (Powell and Baker, 1987), amphibian (Andersen *et al.*, 1987), insect (Schoofs *et al.*, 1987), and dogfish (Vallarino *et al.*, 1989). The C-terminal sequence of NEI, Pro-Ile-NH<sub>2</sub>, is similar to the Pro-Val-NH<sub>2</sub> found in  $\alpha$ -MSH and pre-adsorption of  $\alpha$ -MSH antisera with NEI or even Pro-Ile-amide prevents labelling of the  $\alpha$ -MSH-like peptide in MCH neurons (Nahon *et al.*, 1989) suggesting that  $\alpha$ -MSH immunoreactivity is attributable to cross reaction with the cleaved prohormone. In some cases this cross reactivity only occurs in particular MCH neurons and nerve terminals, for example in the amphibian brain, only the preoptic group of MCH neurons stain with  $\alpha$ -MSH antiserum whereas the infundibular neurons do not (Andersen *et al.*, 1987) and in the carp some secretory granules stain with

both  $\alpha$ -MSH and MCH antisera whilst others are only MCH immunoreactive (Powell and Baker, 1987).

Apparent colocalisation of irMCH with immunoreactive corticotropin releasing factor (CRF) and human growth hormone-releasing factor (hGRF) has also been observed in the rat (Fellmann *et al.*, 1985; Antoni and Linton, 1989) and human (Bresson *et al.*, 1987 - reviewed in Nahon, 1994). Again, the C-terminal amide of CRF is similar to that of NEI and NGE shares a five amino acid stretch within residues 30-37 of hGRF. This labelling of MCH neurons with CRF or hGRF antisera can be extinguished by preadsorption with NEI or NGE respectively (Nahon *et al.*, 1989). Thus, it seems likely that processing of the prohormone varies in different irMCH cell groups and that this has come to light by the artefactual findings resulting from the use of polyclonal antisera.

#### 1.4 MCH receptors

The isolation and characterisation of MCH receptors would be of considerable advantage in the study of this peptide, allowing target sites to be identified. This has proved elusive since radioiodination of tyrosine residues renders the peptide biologically inactive (Baker *et al.*, 1985b) but recently a highly tritiated form of MCH, whilst retaining only ten percent biological activity, has illustrated the presence of MCH receptors in the rat hippocampus, hypothalamus and adrenal gland (Drozdz and Eberle, 1995). In addition, a human MCH analogue, with amino acid substitutions Tyr<sup>13</sup> to Phe and Val<sup>19</sup> to Tyr, retains biological activity when monoiodinated and has revealed high-affinity ( $K_D$  of  $1.18 \times 10^{-10}$  M) binding

sites on mouse G4F-7 melanoma cells, numbering over a thousand receptors per cell (Drozdz *et al.*, 1995).

### 1.5 MCH distribution and colour change function in teleost fish

Many fish and amphibians can camouflage themselves by matching the shade or colour of their skin to their changing environment. This effect is achieved by the dispersal or aggregation of pigment granules within specialised skin cells, collectively called chromatophores. The chromatophores are named for the colour of pigment they contain, for example, those containing white pigment are named leucophores and those containing black pigment, melanophores.

Melanogenesis and melanin dispersal are under the control of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH), released from the pituitary *pars intermedia*. In elasmobranch fish and amphibians, changes of skin colour are achieved by varying amounts of circulating  $\alpha$ -MSH, but in teleost fish colour change comes under the dual hormonal control of  $\alpha$ -MSH and MCH, as well as the sympathetic nervous system. This colour change function of MCH has been extensively reviewed (Baker, 1991; Baker, 1993) and will be dealt with briefly here.

In chum salmon, rainbow trout (Naito *et al.*, 1985), carp (Bird *et al.*, 1989), molly (Batten and Baker, 1988) and tilapia (Gröneveld *et al.*, 1995a) the principle group of magnocellular neurons lie ventrally and bilaterally, in the *nucleus lateralis tuberis pars lateralis* (NLT) near the pituitary stalk, with axons coursing predominantly to the neural lobe of the pituitary gland, and terminating near major blood vessels, giving access to the circulation (Naito *et*



*al.*, 1985). In trout, some fibres penetrate the basement membrane between the neural and intermediate lobes, making direct contact with *pars intermedia* cells (Batten and Baker, 1988) and a few are found in the *pars distalis* close to the pituitary cells (Powell and Baker, 1987). Other axons from the NLT extend dorsally to various parts of the brain including the preoptic area and probably the pretectal region (Naito *et al.*, 1985). In trout, a second, smaller group of irMCH parvocellular neurons has recently been detected bilaterally, near the dorsal surface of the lateral ventricular recess, with axons extending dorsally into similar regions to that of the NLT neurons and ventrally towards the ependymal layer of the third ventricle. These have been termed LVR-MCH neurons (Baker *et al.*, 1995). In addition, in the rainbow trout, between these two nuclei are a small number of large, spider-like cells whose function is as yet unknown (Suzuki *et al.*, 1995).

The development of a solid phase MCH radioimmunoassay (Kishida *et al.*, 1989) for the measurement of plasma MCH, confirmed earlier hypotheses that when a teleost moves against a light coloured background, hypothalamic and pituitary stores of MCH are depleted and plasma MCH concentrations rise as peptide is released into the circulation (Rance and Baker, 1979; Barber *et al.*, 1987; Powell and Baker, 1988). The peripheral effect of MCH is to cause melanin concentration within the dermal and epidermal melanophores, leading to the appearance of pallor, an action directly antagonistic to the darkening effects of  $\alpha$ -MSH (Baker, 1986; Baker, 1988). Interaction between the two peptides also occurs centrally, since MCH depresses the release of  $\alpha$ -MSH from the *pars intermedia*, probably by acting in a paracrine manner on the melanotrophs (Baker *et al.*, 1986; Barber *et al.*, 1987). Interestingly, although the LVR neurons do not appear to send axons to the pituitary, they nevertheless respond positively to a change of background colour since, in

both groups, the MCH cell nuclei are approximately twice the cross-sectional area in white reared trout than in black reared trout, an indication of synthetic activity. Additionally, white reared trout have approximately fourfold more MCHmRNA, in both the NLT and LVR-MCH neurons, than black reared animals (Suzuki *et al.*, 1995). In the teleost tilapia, however, transfer to a white coloured tank elicits a threefold increase in NLT ppMCH mRNA but does not affect the LVR neurons (Gröneveld *et al.*, 1995a)

### **1.6 MCH as a modulator of stress in teleosts**

In vertebrates, chronic stress activates a hormonal cascade. After the stress is perceived, there follows a release of hypothalamic corticotrophin releasing hormone (CRH) which, in turn, stimulates the release of adrenocorticotropin releasing hormone (ACTH), from the anterior pituitary. This hormone circulates to the interrenal gland in fish (adrenal gland in other animals) and causes the secretion of corticosteroids. Although the short term effects of steroids are beneficial to the organism under stress, prolonged release is deleterious and can suppress the normal immune defence system and lead to impaired growth and reproductive activity. A negative feedback system of corticosteroids on the pituitary, CRH neurons and hippocampus restrains overactivity of the system.

While studies were in progress on the effects of MCH in colour change, it was observed that after a mild stress, trout adapted to white tanks maintain lower plasma cortisol concentrations than do black adapted fish (Baker and Rance, 1981) whilst the basal levels are not significantly different (Gilham and Baker, 1984). This implies, therefore, that high levels of MCH, associated with the white tank colour, might suppress the stress-induced

## Chapter one

cortisol release from the trout interrenal. Whilst MCH does not inhibit cortisol release directly (Green *et al.*, 1991) its influences at hypothalamic and pituitary level have been demonstrated. Thus, if trout hypothalami are incubated *in vitro* with sMCH antisera, the CRF-like bioactivity is significantly enhanced (Green *et al.*, 1991). Salmonid MCH also reduces the *in vitro* release of ACTH in a dose dependent manner (Baker *et al.*, 1985a) and, indeed the pituitaries from stressed, black-adapted fish release more ACTH than those from stressed, white-adapted fish (Baker *et al.*, 1986).

In white-adapted trout, a single injection stress raises plasma cortisol levels but does not affect MCH release, whereas repetitive stress causes a ninefold increase in plasma MCH content, an effect prevented by treatment with the synthetic steroid, dexamethasone (Green and Baker, 1991). Assessment of ppMCH synthesis, by measurement of radiolabelled methionine uptake, shows that a mild, daily stress is stimulatory but that a more moderate, daily handling and confinement, stress results in a reduction of MCH synthesis (Baker and Bird, 1992). Similarly, a one minute, repeated cold stress stimulates MCH mRNA in trout fry (Suzuki *et al.*, 1996).

To summarise, in fish, MCH has an inhibitory effect on ACTH and/or CRH release, leading to a reduction in cortisol levels. A mild, daily stress increases ppMCH synthesis, whereas a more severe, daily stress results in a reduction of ppMCH synthesis. Cortisol exerts a negative effect on MCH release but its effects on synthesis are as yet unknown.

### 1.7 MCH as a modulator of stress in rats

As in fish, the synthesis of rat ppMCH is also influenced by stress, but seemingly in the opposite way. Thus chronic footshock stress for one day provides a significant inhibitory effect on rMCH mRNA expression, a response which is attenuated after three days and is no longer apparent after a week (Presse *et al.*, 1992). The restoration of mRNA levels to that of controls may be due to a positive, stimulatory effect of the rising levels of corticosteroids which contrasts with their negative, feedback effects on CRH and ACTH gene activity (Jingami *et al.*, 1985). In confirmation, adrenalectomy results in a reduction of MCH mRNA hypothalamic content, which can be corrected by dexamethasone (Presse *et al.*, 1992). The addition of CRH to rat hypothalamic cultures depresses MCH and NEI synthesis and secretion, but dexamethasone increases the cell content of both peptides in a dose dependent manner and increases MCH secretion (Parkes and Vale, 1992).

In one study (Baker *et al.*, 1985a) a high (10 nM) dose of sMCH reduced the CRH-induced release of ACTH in rat pituitary fragments, but did not affect basal levels. Other workers have been unable to confirm these results since neither sMCH nor rMCH affect ACTH or CRH release *in vitro* (Nahon *et al.*, 1989; Navarra *et al.*, 1990). Conversely, it was shown that rMCH has a stimulatory effect on ACTH secretion in some *in vitro* systems (Nahon *et al.*, 1989) and rMCH injected either centrally or peripherally, after permeabilisation of the blood brain barrier, leads to a significant enhancement in ACTH release *in vivo* (Jezova *et al.*, 1992). On the other hand, a recent study shows that icv administration of rMCH, but not NEI, at a time corresponding to the peak of the circadian rhythm of ACTH, leads to a decrease in both basal and stress induced ACTH release (Bluet-Pajot *et al.*, 1995). Co-

administration of both peptides before the stress prevents this inhibitory action of MCH, suggesting that NEI can be an MCH antagonist, were both peptides to be released in synchrony.

In summary, MCH has a stimulatory effect on ACTH release when given in the morning, at the beginning of the sleep phase but, in another study, MCH was shown to decrease ACTH levels at the time of their circadian peak. Stress and adrenalectomy depress MCH mRNA synthesis and glucocorticoids positively regulate MCH mRNA synthesis and peptide release. A representation of findings in both fish and mammals is shown in Figure 3.

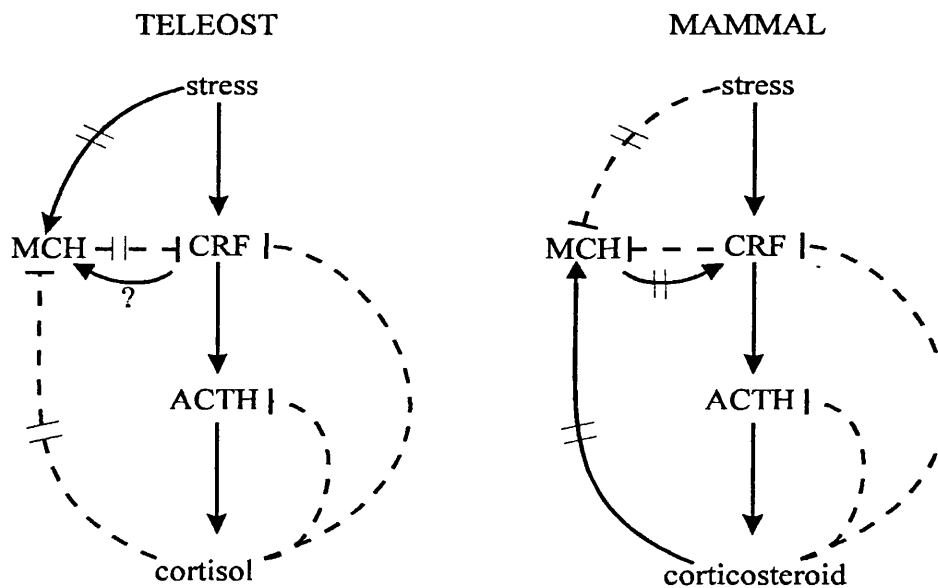


Fig 3 Representation of a speculative relationship between MCH and HPI (HPA in mammals) axis. Broken lines indicate an inhibitory effect and solid lines indicate a stimulatory influence. A slashed pathway indicates that the route of action is unknown (taken from Baker, 1994).

The conflicting evidence does not make clear the role of MCH or related peptides in the modulation of the stress response. The mechanisms in fish and mammals appear to operate along different pathways, or it could be that the circadian rhythmicity of the hormones involved in the stress response determine the observed interaction between them at any given time. Why the MCH peptide, with such a highly conserved structure should, apparently, have evolved opposing effects in fish and mammals remains to be clarified.

### **1.8 MCH distribution and antagonism with $\alpha$ -MSH in rat brain**

Immunocytochemical (Naito *et al.*, 1988; Skofitsch *et al.*, 1985; Zamir *et al.*, 1986) and *in situ* hybridization (Bittencourt *et al.*, 1992; Presse *et al.*, 1992) studies of the location of MCH in rat brain have, together, shown that the cell bodies are distributed throughout the mid and caudal regions of the dorsolateral hypothalamus. Anteriorly, they extend from the paraventricular nuclei, and surround the fornix and medial forebrain bundle. Caudally, they are located in the sub-zona incerta region, above the ventromedial nucleus and dorsomedial to the optic tract. Fibres from these perikarya form an extensive network that projects to most regions of the brain and spinal cord. A few cell bodies are also found in the olfactory tubercle and pons but they represent only five percent of the total neuronal system (Nahon *et al.*, 1993).

The widespread distribution of irMCH fibres in the rat brain suggest that the peptide influences many, independent brain activities. Just as the studies on stress in fish led to comparative studies in mammals, the concept of antagonism between  $\alpha$ -MSH and MCH on fish melanophores has provided a basis for investigations into similar antagonism in the

mammalian brain. Four examples of such antagonism have been demonstrated, and are detailed below. All might be described as behavioural responses, thus according with the belief that the areas in which the irMCH perikarya are located, the lateral hypothalamus (LH) and zona incerta (ZI) in the rat brain, are implicated in behavioural effects (reviewed in Nahon, 1994).

The first example concerns auditory gating, a classical conditional-test suppression paradigm. When paired auditory stimuli are played 500ms apart, the second tone-evoked potential, which can be measured in the hippocampus, is of lower amplitude than the first. When  $\alpha$ -MSH is infused into the rat brain the magnitude of the first, conditioning, response is increased but if MCH is administered the conditioning response is diminished and, if given before  $\alpha$ -MSH, will abolish this stimulatory effect (Miller *et al.*, 1993). This result implies that MCH reduces the probability of a behavioural change in response to a novel sound. In a similar way, rats exhibit certain behavioural traits, such as grooming stretching and yawning in a variety of social contexts, including exposure to novelty under stress, or after a meal (Spruijt *et al.*, 1992). Whilst icv injection of  $\alpha$ -MSH will induce excessive grooming in the rat, prior administration of MCH diminishes this response (deGraan *et al.*, reviewed in Eberle, 1988). Alpha MSH treatment also delays extinction of the passive avoidance response whilst MCH has the opposite effect (McBride *et al.*, 1994) and, finally, preadministration of MCH antagonises the increased aggressive and reduced exploratory behaviour caused by  $\alpha$ -MSH whilst having no effect when given alone (Gonzalez *et al.*, 1996). These results suggest that  $\alpha$ -MSH improves learning and attention, but MCH could be said to encourage dearousal.

## 1.9 The effects of osmotic stimuli on MCH in rat

The mapping of a modest number of MCH fibres in the median eminence and pituitary stalk of the rat prompted an early investigation into the possibility that MCH may be involved in the hypothalamo-hypophyseal system and the control of posterior pituitary function. An osmotic stimulus of 2% salt in drinking water for 5d, associated with enhanced secretory activity from the neurohypophysis, also causes significant increases in MCH-like immunoreactivity in the lateral hypothalamus and neurointermediate lobe, which could be interpreted as a cessation of MCH release (Zamir *et al.*, 1986). More recently, *in-situ* hybridisation and Northern blotting have shown that most of the hypothalamic rMCH mRNA-containing neurons show a threefold decrease in MCH message during salt loading of up to six days (Presse and Nahon, 1993). However, a few neurons in the zona incerta and fornix respond by an increase in message, and those in the medulla and pons do not respond at all, which illustrates a differential regulation of MCH gene expression with this stimulus.

A twenty-four hour withdrawal of water also causes a dramatic decrease in rMCH mRNA in female rats, but has variable results in males, suggesting a sex-specific heterogeneity in MCH gene regulation (Presse and Nahon, 1993). Since, generally, hyperosmolarity and dehydration cause a similar depression in MCH synthesis and release, the peptide may function to stimulate diuresis or inhibit water intake in rats.



### **1.10 MCH and feeding behaviour in rodents**

Although it has long been established that some of the products of the stress response, for example CRF and corticosteroids, have an influence on appetite (reviewed in Morley, 1987) it is only recently that MCH has been investigated in this respect. The localisation of MCH neurons and fibres in the rat brain suggest that the peptide may participate in the control of food and water intake, but the results with regard to feeding behaviour are contradictory.

One group has recently shown, in rats, that food deprivation for 24 h and 48 h leads to a significant rise in MCH mRNA which is attenuated after 72 h. Additionally, an icv injection of 1-100 ng MCH reduces food consumption within 2 h of administration (Presse *et al.*, 1996), a result confirmed in obese and normal mice after 24 h fasting (Qu *et al.*, 1996). However, in the latter study, 5 µg MCH given by catheter into the lateral ventricles of rat brain led to a doubling of calorific intake, in apparent contradiction to the earlier findings, and due, perhaps, to the use of a pharmacological, rather than physiological, dose.

### **1.11 MCH in humans**

The identification of MCH in rat brain inevitably led to investigations of the peptide's presence in the human brain. An early immunocytochemical study showed irMCH cell bodies in the hypothalamus, in the periventricular area, and fibres in that region, extending to the median eminence and pituitary stalk (Pelletier *et al.*, 1987). There are three pro-MCH genes in the human genome, an authentic form located on chromosome 12q23-q24 and two variant forms on chromosome 5p14 and 5q12-q13 (Pedetour *et al.*, 1994). The authentic and variant genes are very similar, suggesting only a recent divergence (Breton *et al.*,

1993b) but, in structure, one variant form differs from the authentic hMCH by the substitution of four hydrophilic or positively charged amino acids for hydrophobic residues, suggesting different conformation and, perhaps binding, properties.

The chromosomal loci of the two pro-MCH genes have both been associated with human ailments. The authentic hMCH gene is located near the gene for Darier's disease, which has recently been linked with manic depression (Ewald *et al.*, 1994) and the variant hMCH gene is close to the putative schizophrenia locus at 5q11.2-13.3 (Sherrington *et al.*, 1988). Whether this indicates functional linkage has not been investigated.

### **1.12 Aim of the present study**

Although early work on MCH centred on fish, in which the peptide was discovered, more recent studies have focussed on mammals. This is a natural progression, fuelled undoubtedly by the desire to discover the role and possible value of MCH in humans. However, many gaps exist in our knowledge of MCH function in lower vertebrates; for example only one published work exists on mapping the peptide for either amphibians, reptiles or insects. Since MCH was first characterised in a fish, and early observations in these animals gave direction to the pursuit of understanding MCH function in mammals, it is self evident that studies in lower vertebrates are of great help in contributing to our understanding in higher vertebrates.

Appreciation of the general importance of a peptide function would be most emphasised if that function can be shown to be exhibited in a wide range of vertebrate classes. For this

reason it seemed relevant not only to look at the ontogeny of MCH in a lower vertebrate but also to determine if the environmental factors recently shown to influence MCH mRNA expression in mammals hold true in other animal classes, thus revealing a long history of association of MCH with particular physiological challenges.

This thesis describes an investigation into MCH function in amphibians and fish, starting with the ontogeny of the peptide in the terrestrial frog, *Rana temporaria*, and compares the activity of MCH neurons between this and a strictly aquatic species, *Xenopus laevis* at the time of late metamorphosis, when the grass frog emerges onto land for the first time. The MCH immunoreactivity was next mapped in the adult brain of *Rana temporaria* in wax-embedded and frozen sections. This work reveals that a hitherto unseen group of MCH neurons become visible in the female amphibian telencephalon at a time when the animal is carrying mature eggs and, furthermore, that the neurons in another locus, the pre-optic nucleus, not previously associated with *R. temporaria*, become visible only during this period. An investigation into the response of amphibian MCH neurons to salinity exposure identifies a heterogeneity in the reaction between different cell populations and the suggestion of a sexually dimorphic response to this physiological stress.

A study of the activity of trout hypothalamic MCH mRNA on exposure to differing strengths of seawater shows that a moderate salinity leads to a stimulation of one MCH neuronal population but that full strength seawater depresses the message in both irMCH groups. It is suggested that a feedback system may operate, since mRNA values were restored to that of controls within a few days. Finally, because brain peptides are so often expressed in the gut, peripheral MCH was investigated in three regions of the trout gastro-

## Chapter one

intestinal tract with the result that immunoreactive MCH was visualised in the pyloric region of the gut in cells that, by their morphology, appeared to be similar to the mast cells of the mammalian immune system.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 Animal husbandry

##### 2.1.1 Trout

Rainbow trout, *Oncorhynchus mykiss*, of approximately 200-300 g body weight, were obtained from Alderley Trout Farm, Wootton-under-Edge, Gloucestershire. Some fish were also home-reared. The fish were kept at a maximum density of twelve in 250 litre black fibreglass tanks, supplied with a constant flow of tap water. Commercial trout pellets were given once daily in a quantity equivalent to 1 % body weight (maintenance diet) per fish. The aquarium was kept at a constant temperature of 11 °C and photoperiod of 16.5 h light and 8.5 h dark (lights off 2230-0630 h). Fish were acclimated to these conditions for a minimum period of 10 days before use.

##### 2.1.2 Frogs

Adult *Rana temporaria* (mean weight  $18 \pm 2$  g) were obtained from Blades Biological Ltd. and housed in 20 litre grey plastic tanks with approximately 1 cm fresh water in the base and a constant trickle feeding through. Rocks, grass and moss were provided for cover. These tanks were housed in the aquarium as above. Food was initially offered but declined and so, thereafter, not given. Tadpoles were caught locally and reared to adulthood in tanks of fresh water at room temperature (c. 20°C) and in natural daylight. They were fed with powdered trout pellets.

### **2.1.3 Toads**

*Xenopus laevis* tadpoles were obtained from Blades Biological Ltd, and reared past metamorphosis in plastic 10 litre tanks, filled with dechlorinated tap water. The tanks were housed in a room heated to 22 °C and with a 16 h light : 8 h dark photoperiod (lights off 2200-0600 h). As adults, the toads were fed twice weekly, *ad libitum*, with minced heart or liver.

## **2.2 Collection of trout blood and plasma extraction**

Trout were deeply anaesthetised in 0.06 % (v/v) phenoxyethanol in water. The tail area was washed in clean water and dried on paper towelling. Blood from the severed peduncle was collected in a 4 ml polypropylene tube containing 50 µl ice cold 6 % (w/v) sodium EDTA as anti-coagulant. The samples were centrifuged at 3000 g for 15 min and the plasma supernatant was stored at -20 °C until required.

## **2.3 Collection of brain tissue**

### **2.3.1 Trout**

Following anaesthesia, as above, the head was removed and the top of the brain case cut away to expose the dorsal surface of the brain. Fixative (4 % paraformaldehyde) was then injected into the brain ventricles via the optic tecta. The brain and pituitary gland were removed and postfixed for 24 h at 4 °C.

### **2.3.2 Amphibia**

Animals were anaesthetised in 0.06 % (v/v) phenoxyethanol in water, and then perfused via the aorta with ice cold, heparinised phosphate buffered saline (PBS 0.01 M; pH 7.6) followed by approximately 100 ml Bouin's fixative. After removal, the brain tissue was postfixed for 24 h at 4 °C. In some experiments adequate fixation was achieved by perfusion alone. In this case, following anaesthesia and severing of the spinal cord, the upper palate was removed to expose the underside of the brain. Fixative was then injected in and around the brain before removal and postfixation.

## **2.4 Immunocytochemistry**

### **2.4.1 Wax embedded sections**

Fixed brain tissue was dehydrated in an ethanol series, cleared in xylene and embedded in paraplast wax blocks. Serial sections of 5 µm or 10 µm thickness were mounted on glycerine-albumen coated slides. Immunoreactive MCH was demonstrated by the peroxidase anti-peroxidase method (Sternberger, 1974). Phosphate buffered saline (PBS 0.02 M; pH 7.6) was used throughout, both for the dilution of antisera and for the rinsing of samples.

Sections were hydrated, washed in PBS for 15 min and then incubated for 30 min with 3 % (v/v) lamb serum (30 µl serum in 1 ml PBS) to reduce non-specific binding. For the PAP procedure, sections were incubated overnight in humid chambers, at room temperature, with anti-salmonid MCH (Eberle) developed in rabbit, diluted x1000 in PBS containing 3 % (v/v) lamb serum (LS). After thorough washing in PBS, sections were incubated for 1 h in goat anti-rabbit globulin (Sigma Chemical Co., Poole, U.K) diluted x25 in PBS with 3 % (v/v) LS. After rinsing and two 15 min washes in PBS, sections were then incubated for 1h in

rabbit peroxidase anti-peroxidase (Sigma) diluted x200 in PBS with 3 % (v/v) LS, and then washed and soaked for 15 min in Tris HCl buffer (Tris 10 mM; pH 7.6). To visualise the MCH antibody-antigen complex, tissues sections were soaked for between 10 and 15 min in freshly prepared 3,3'-diaminobenzidine tetrachloride (DAB, Sigma) solution (0.025 g DAB in 100 ml Tris HCl buffer) with 100  $\mu$ l 30 % stock  $H_2O_2$ . Following counterstaining in haematoxylin sections were dehydrated, cleared and mounted with DPX.

#### **2.4.2 Vibratome sections**

Brain tissue, fixed in Bouin's fluid, was thoroughly rinsed in 0.01 M PBS (pH 7.5) to remove as much fixative as possible and then attached to a vibratome chuck with Superglue. The tissue was then coated in molten glycerin-gelatin (15 ml glycerol, 16 g gelatin in 70 ml distilled water) and briefly put on ice to set. Sections were cut at 100  $\mu$ m and collected in ice cold PBST (0.01 M PBS with 0.2 % v/v Triton-X 100; pH 7.5).

Sections were immunostained using the biotin-streptavidin method with a commercial kit (Vectastain ABC Elite, Vector Laboratories, USA). The free floating sections were soaked in 1 % (v/v)  $H_2O_2$  in PBS, to block endogenous peroxidase activity, rinsed thoroughly in PBST and then incubated for 30 min in 3 % (v/v) normal goat serum (GS) in PBST. Sections were then incubated at 4 °C in 837 anti-salmonid MCH, diluted x5000, in 0.01 M PBST with 3 % (v/v) GS for 48 h. After thorough rinsing in PBST, sections were incubated for 1 h in biotinylated goat anti-rabbit globulin (5  $\mu$ l in 1 ml PBST with 15  $\mu$ l GS) and then washed in PBST. Sections were then incubated in biotin-streptavidin conjugate for 1 h. After rinsing in PBST and 0.05 M Tris HCl buffer (pH 7.5) sections were soaked, first for 15 min in DAB solution (25 mg DAB in 100 ml Tris HCl buffer) and then in fresh DAB



solution with added  $\text{H}_2\text{O}_2$  (100  $\mu\text{l}$  of 30% stock per 100 ml Tris HCl buffer). When visualisation was complete, sections were rinsed in PBS and mounted onto gelatin coated slides. After 24 h, the sections were dehydrated, cleared and mounted in DPX.

## **2.5 Cortisol radioimmunoassay**

### **2.5.1 Preparation of steroid**

Liberation of the cortisol from blood plasma was achieved by precipitation of the carrier proteins in ethanol. Duplicate 100  $\mu\text{l}$  aliquots of plasma samples were dispensed into 4 ml polypropylene tubes to which was added 500  $\mu\text{l}$  absolute ethanol. After mixing, a further 500  $\mu\text{l}$  of ethanol was added to each tube and these were then centrifuged at 3000 g for 15 min. Appropriate aliquots (100  $\mu\text{l}$ , 200  $\mu\text{l}$  or 400  $\mu\text{l}$ , depending on the anticipated cortisol concentration) were dispensed into fresh tubes and dried under vacuum for 2 h. The contents were then resuspended in 200  $\mu\text{l}$  PBSG (0.05 M PBS pH 7.4, with 0.1 % (w/v) gelatine) containing  $^3\text{H}$  and cortisol antiserum (see 2.5.4), the same quantity of which was added to duplicate 10  $\mu\text{l}$  aliquots of a range of cortisol standards, from 12.5 pg to 1600 pg (see 2.5.3). The tubes were then incubated at 4 °C overnight.

### **2.5.2 Separation of bound and unbound fractions**

Following overnight incubation, 500  $\mu\text{l}$  ice cold dextran charcoal mixture (0.125 g Dextran and 0.5 g charcoal in 100 ml PBSG) was added to each sample tube. After 15 min, samples were centrifuged at 4 °C at 3000 g for 15 min. The supernatant was carefully decanted into scintillation vials, each containing 6 ml scintillation fluid (Optiphase Safe, Wallace Scintillation Products Ltd; UK). Samples were counted on a scintillation counter and the cortisol concentrations calculated by reference to a standard curve.

### **2.5.3 Cortisol standards**

A stock solution of standard cortisol was prepared by dissolving 3.2 mg synthetic cortisol (Hydrocortisone, Sigma) in 20 ml absolute ethanol (ie. 1.6  $\mu\text{g}/10\ \mu\text{l}$ ). Working standards were prepared by diluting 20  $\mu\text{l}$  stock solution in 20 ml ethanol (1600 pg in 10  $\mu\text{l}$ ) and, thereafter, serial dilutions were prepared down to 12.5 pg in 10  $\mu\text{l}$ . Standards were stored at -20 °C until use. For preparation of the standard curve, duplicate 10 $\mu\text{l}$  aliquots were used and treated as for the sample extracts.

### **2.5.4 Tritiated cortisol and cortisol antibody**

The  $^3\text{H}$ -(1,2,6,7)-cortisol (Amersham, 250  $\mu\text{Ci}$  in 250  $\mu\text{l}$  toluene/ethanol, 9:1 solution) was diluted in 25 ml toluene/ethanol and stored at -20 °C. For use, a 100  $\mu\text{l}$  aliquot of stock was evaporated to dryness and resuspended in 20 ml PBSG. The cortisol antiserum (Cortisol R5, raised by Dr I Gilham, Bath University) was stored at -40 °C as a x10 plasma dilution in PBSG. A 200  $\mu\text{l}$  aliquot was added to the beaker containing  $^3\text{H}$ -cortisol in PBSG immediately before adding 200  $\mu\text{l}$  to each assay tube.

## **2.6 *In-situ* hybridization**

### **2.6.1 Preparation of slides**

Slides were soaked in dilute Teepol (BDH) and warm water for 3h and then passed through a series of distilled water baths, and placed in a bath of subbing mixture (2.25 g gelatin, 0.23 g chromic potassium sulphate in 800 ml distilled water) for 2 min. After drying overnight the slides were again re-immersed in subbing mixture and left to dry, after which they were stored at room temperature in dust-free boxes.

### 2.6.2 3' End-labelling of oligodeoxynucleotide probe (MCH2)

The following procedure produced approximately 50 µl labelled MCH2 probe.

Using sterile tips the following were mixed in an Eppendorf tube :

27 µl MilliQ water

10 µl 5x Tdt buffer (Boehringer Mannheim)

5 µl CoCl<sub>2</sub> (Boehringer Mannheim)

5 µl <sup>35</sup>SdATP (NEN)

1 µl 5 µM MCH2 probe (~ 42ng. See p 78 for probe details)

After thorough mixing, 1 µl cold terminal transferase (Tdt, Boehringer Mannheim) was added and this was then incubated in a water bath at 37 °C for 1h. The Eppendorf tube was removed from the water bath and had the following additions :

250 µl TE buffer (10mM Tris-Cl, 1mM EDTA)

2 µl yeast tRNA (Boehringer Mannheim)

250 µl phenol chloroform isoamyl alcohol (Sigma)

The contents were thoroughly mixed and the tube was centrifuged at 14,000 g for 5 min. after which, the upper phase was removed and pipetted into a clean Eppendorf tube containing 300 µl chloroform/3-methyl-1-butanol (previously mixed in the ratio 490 µl : 10 µl). The tube was again centrifuged at 14,000 g for 2 min and the upper phase removed and dispensed into a clean Eppendorf tube, containing 15 µl 4M NaCl, to which was then added 750 µl 100% ethanol. The tube was inverted to mix the contents and incubated upright at -80 °C for 15 min. after which, the tube was centrifuged at 14000 g for 15 min. The supernatant was removed (into radioactive waste) and the pellet was pushed to the bottom of the tube by brief centrifugation and 50µl TE buffer was then added. To check the activity of the labelled probe, duplicate 1µl samples were dispensed into a polypropylene

vial containing 10 ml scintillation fluid and read on a scintillation counter. The CPM readings were recorded.

### **2.6.3 Purification of the labelled probe using a QIAquick kit**

In the second *in-situ* hybridization experiment (Chapter 4) the labelled probe was purified using a QIAquick kit (QIAquick nucleotide removal kit 28304, Quiagen, Hilden, Germany). This method replaced all the steps following the 37 °C incubation of the labelled probe :

After removal of the Eppendorf tube from the water bath, 500 µl of PN buffer (QIAquick kit) was added and the contents were transferred to a spin column, which was placed within a 2 ml centrifugation tube. The contents were centrifuged at 6,000 g for 1 min and then the column was transferred to a fresh tube, had 500 µl PE buffer (QIAquick kit) added and was centrifuged again at 6,000 g for 1 min. This step was then repeated, after which the contents of the tube were discarded. The column was replaced into the same tube and centrifuged at 14,000 g for 1 min. It was then placed into a 1.5 ml hinged Eppendorf and had 50 µl MilliQ water carefully added, such that the resin filter at the base of the column was covered. To elute the probe, the Eppendorf was centrifuged at 14,000 g for 1 min. The column was then discarded and the purified probe stored at -20 °C until use.

### **2.6.4 Hybridization of tissue sections**

Wax-embedded sections (10 µm) were mounted onto coated slides, dried for 24 h at 37 °C, then rehydrated and immersed in autoclaved MilliQ water. The slides were then transferred to sterile Coplin jars containing 2xSSC (see Appendix) which were pre-warmed, in a water bath, to 65 °C. After 10 min the slides were put into fresh, sterile Coplin jars containing

## Chapter two

autoclaved MilliQ water and left until required. Labelled probe was diluted with autoclaved MilliQ water, to give a final activity of  $1 \times 10^7$  CPM ml<sup>-1</sup> and then the total required quantity was divided into Eppendorf tubes, each containing 900 µl hybridization buffer (see Appendix) and 20 µl DTT (dithiothreitol, Sigma). The number of tubes so prepared was dependent on the number of tissue sections, such that each slide containing x8 trout brain sections required 90µl hybridization mixture.

Each slide was taken from MilliQ water and excess moisture was removed with clean paper towelling. Hybridization mixture (90 µl per slide) was pipetted over the sections, which were then covered with a strip of Nescofilm (Nesco, Nippon Shoji Kaisha, Ltd; Japan). The slides were incubated overnight in humid chambers at 37 °C.

### **2.6.5 Post hybridization washing**

Each slide was immersed in 1xSSC, at room temperature, to remove the Nescofilm cover slip. Slides were then passed through four brief washes of 1xSSC and further washed in four changes of 1xSSC at 60 °C, each for 15 min. The slides were then washed twice in fresh 1xSSC, at room temperature, each for 30 min. After a final rinse in distilled water, the slides were dried in an incubator at 37 °C.

### **2.6.6 Autoradiography**

Slides were exposed to autoradiographic film (Hyperfilm-MP, Amersham International plc, UK) for variable periods of time (see individual experiments) together with autoradiographic micro-scales, of high (maximum 2 nCi) and low (maximum 88 pCi) <sup>14</sup>C standards (Amersham International, UK). The films were then developed and fixed. Quantification

(total binding) of the MCH signal was obtained by measuring the area and optical density of a signal on an image capture system. The computer system allowed the background signal to be subtracted from the image and a threshold of density to be set by the operator. Following the setting up of those parameters, the hybridization signal in each macroscopic brain region area was measured, and by reference to the standards of known radioactivity, a third degree polynomial curve was constructed against which subsequent readings were applied. Binding was calculated by multiplying the optical density and area readings together, and the readings from each section were then summed together to give a total binding for each animal.

#### **2.6.7 Dipping slides in photographic emulsion**

In order to photograph selected sections from the *in-situ* experiments, the MCH signal had first to be visualised, for which purpose slides were dipped in photographic emulsion and developed. The dipping emulsion was prepared, in a darkroom, by mixing the following and heating to 45 °C in a water bath :

30 µl 50 % (v/v) glycerol in DEPC-treated water

6 ml MilliQ water

made up to 10 ml with emulsion (nuclear research emulsion, Ilford, UK)

Slides were coated in the emulsion and left to dry before being put into light-proof wrapping and stored, with a desiccant, for a period of three weeks. The slides were then put into developer (D-19, Kodak) for 3.5 min, stop bath (Kodak, UK) for 30 s and fix (Unifix, Kodak) for 3.5 min. After being thoroughly washed in water, the sections were dehydrated in an ethanol series and stained in toluidine blue before being cleared in xylene and mounted in DPX.

## CHAPTER 3

### DEVELOPMENTAL CHANGES IN MELANIN-CONCENTRATING

### HORMONE IN THE GRASS FROG, *Rana temporaria*.

#### 3.1 Introduction

In both mammals and fish ppMCH mRNA can be detected in brains well before birth, or hatching, so the peptide is formed, ready for secretion, before the initiation of free life. In trout MCH mRNA is detectable in the NLT region seven days before hatching (Suzuki *et al.*, 1996) and in the rat, MCH mRNA can first be measured, by Northern blotting, between the thirteenth and eighteenth day of embryonic life (Presse *et al.*, 1992). The irMCH cells, first seen at the fourteenth embryonic day, develop steadily thereafter, but remain relatively inactive, until a few days after birth when both the peptide and mRNA rapidly reach adult levels of activity (Fellmann *et al.*, 1993). In the mouse, a weak mRNA signal is detected five days after birth, the earliest stage examined (Breton *et al.*, 1993a) and human MCH-producing neurons can be localised in the lateral hypothalamus during the seventh week of development (Bresson *et al.*, 1989).

Although MCH immunoreactivity has been detected in the adult amphibian brain (Andersen *et al.*, 1986; Baker, 1988), to date there are no published data on MCH ontogeny in this class of animal. The time when a hormone shows an increase in abundance, or a change in secretion, in response to environmental challenge reflects its physiological involvement and may indicate a potential function. Hence, the present work looks at MCH immunoreactivity in the brain of *Rana temporaria* larvae, from the earliest feeding stage to the emergence of

the froglet onto land. Late metamorphic stages of the South African clawed toad, *Xenopus laevis*, are also examined to provide a comparison between terrestrial and aquatic species.

### 3.2 Materials and methods

#### 3.2.1 Amphibians

Tadpoles were caught locally or obtained from a supplier (Blades Biological Ltd) and maintained as outlined in Chapter 2. *Rana temporaria* tadpoles were staged according to Taylor and Kollross (1946) and examined at six stages (see Table 1) from early feeding larvae to emergent froglets. *Xenopus laevis* tadpoles were staged according to Nieukoop and Faber (1956) and examined in late climax. Anuran tables of comparative staging by morphological events were used (Dodd and Dodd, 1976).

**Table 1**

**Comparison of larval stages of *R. Temporaria* and *Xenopus laevis***

<b>Taylor-Kollross stage</b>	<b>Nieukoop-Faber stage</b>	<b>Developmental features</b>
TK III	NF 49-50*	Premetamorphosis. Early feeding stage. First thyroid follicles formed.
TK XIV	NF 57*	Prometamorphosis. Hindlimbs emerge. Several stages of foot development follow.
TK XX, XXI and XXII	NF 62-63+	Metamorphic climax. Front limbs break through. Tail length to body ratio reduces from 2:1 to 1:1. Larval mouthparts lost. Gill resorption.
TK XXIV	NF 65	Emergence (in <i>Rana</i> ). Small tail stump remaining.

Information taken from Dodd and Dodd (1976). \* not examined



### 3.2.2 Preparation of brain tissue and immunocytochemistry

Animals were anaesthetised in 0.06%(v/v) phenoxyethanol in water. The top of the skull was removed and the area perfused with Bouin's fixative. After removal from the braincase the brains were postfixed in Bouin's overnight. Fixed tissue was dehydrated through an ethanol series, cleared in xylene and then embedded in wax. Sections were cut at 5µm.

Immunoreactive MCH was demonstrated by the peroxidase-antiperoxidase method (Sternberger, 1974) using anti-salmonid MCH antisera raised in rabbit (Andersen *et al.*, 1986; Barber *et al.*, 1987) or anti-rat MCH raised in rabbit (both generously provided by Prof. A.N. Eberle). The specificity of these primary antisera was confirmed by incubating alternate sections with normal anti-sMCH or preabsorbed antiserum (10 µg MCH in 500 µl diluted primary antiserum).

The immunostaining protocol for wax embedded sections, outlined in Chapter 2 (section 2.4.1) was followed. As an indicator of cell synthetic activity, the nuclear areas of irMCH neurons were measured using a computer image scanning device controlled by software developed for the purpose by Mr. T. Stickland. Between thirty and one hundred nuclei were measured, depending on the number of irMCH cells present, in each brain. The resulting data were then analysed statistically using two way ANOVA.

### 3.3 Results

#### 3.3.1 MCH distribution in metamorphic stages of *Rana temporaria*

Immunoreactive MCH perikarya were first observed, with certainty, in *Rana* at mid-metamorphic climax (TK XXII) when the tadpole had fore-limbs and a tail length equivalent to that of the hind legs. The MCH cell bodies were located in the postero-lateral hypothalamus, in the regions of the dorsal and ventral infundibular nuclei (NID and NIV). The cells were small and weakly stained (Figure 4a) with small nuclei. Nucleoli were not always visible and no axonal projections were seen. A few nerve tracts containing immunoreactive granules were visible in the vicinity of the perikarya, but not in other regions of the brain.

At stage TK XXIV, the newly emergent froglet, a very few, small irMCH perikarya were still seen in the NIV, as in stage XXII tadpoles. The group of immunoreactive MCH cells in the NID was now enlarged, containing many more cell bodies, and extended further anteriorly, level with the optic chiasma, showing the adult distribution. In comparison with stage XXII, these cells had increased granular cytoplasm, large darkly stained nuclei, and pale nucleoli (Figure 4b). A few, short axonal projections were visible, but few nerve tracts.

#### 3.2.2 Comparison of MCH neuronal activity in pre- and post-emergent stages

Nuclear areas of irMCH cells in the NID showed a highly significant difference between the mean nuclear areas in TK XXII pre-emergent, aquatic tadpoles ( $27.9 \pm 1.43 \mu\text{m}^2$ ) and TK XXIV post-emergent, terrestrial froglets ( $45.8 \pm 3.7 \mu\text{m}^2$ ;  $P < 0.001$ ). The data are

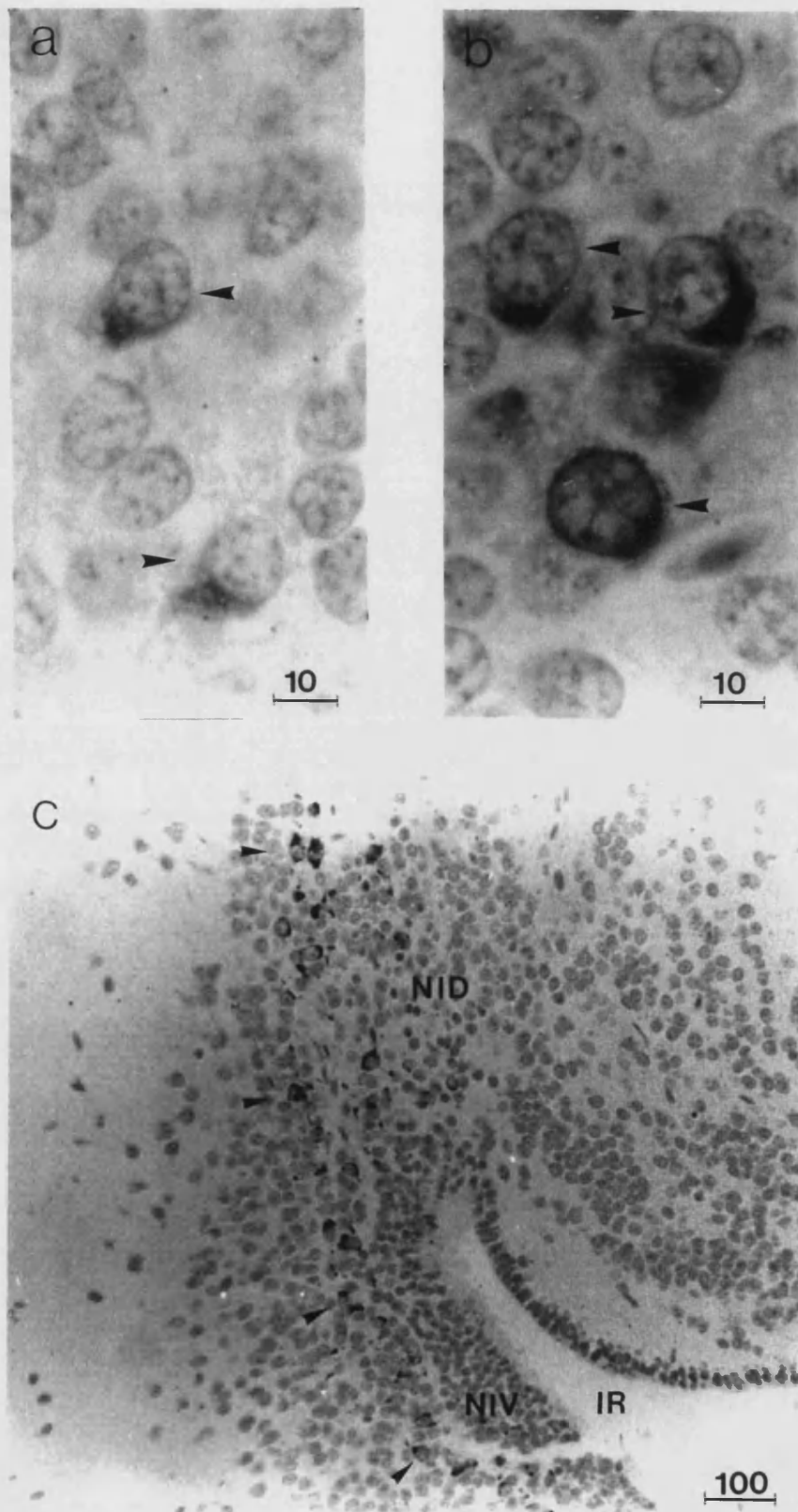


Fig 4. Immunoreactive MCH neurons in the brains of *Rana temporaria* and *Xenopus laevis*. (a) irMCH neurons (arrowed) in the NID region of stage TK XXII tadpoles showing small, palely staining nuclei. (b) irMCH neurons (arrowed) in the NID region of stage TK XXIV froglets showing increased nuclear size and granular cytoplasm. (c) irMCH neurons in the NID and NIV regions of *Xenopus laevis* at stage NF 63. NIV ventral infundibular nucleus, NID dorsal infundibular nucleus, IR infundibular recess. Scale bar represents μm.

summarised in Table 2. As the cumulative numbers of small NIV irMCH cells in both *Rana* tadpoles and froglets were <10, data for these cells are not shown.

**Table 2**  
**Changes in irMCH neuronal cell number and nuclear size**  
**during late metamorphosis**

Developmental stage	Mean nuclear area* $\mu\text{m}^2 \pm \text{SEM}$	Mean total no. of irMCH cells per brain
<i>R. temporaria</i> TKXXII	$27.90 \pm 1.43$	$51 \pm 8$
TKXXIV	$45.75 \pm 3.70$	$210 \pm 31$
<i>X. laevis</i> NF 63, small cells	$22.55 \pm 2.38$	$30 \pm 3$
NF 63, large cells	$33.10 \pm 0.65$	$815 \pm 49$
NF 65, small cells	$22.20 \pm 2.80$	$41 \pm 11$
NF 65, large cells	$33.38 \pm 0.88$	$820 \pm 83$

\* Between 30-100 nuclei were measured per animal. n=3 in each group.

### 3.2.3 Comparison with *Xenopus laevis*.

Similar sets of measurements were made for three *Xenopus laevis* tadpoles (NF 63) and three toadlets (NF 65) of equivalent development to *Rana*. In both stages irMCH perikarya were much more abundant than in the *Rana* tadpoles or froglets (Table 2). In both stages there, again, seemed to be two sizes of irMCH neurons in the infundibular area, with small, palely staining cells located in the NIV, close to the infundibular recess, and a greater number of larger, more heavily stained cells extending anterodorsally, largely contained within the NID (Figure 4c) Nuclear measurements were categorized according to cell size. A two way ANOVA showed that there was no significant difference in nuclear areas

between tadpoles and toadlets in either the small or large cell populations and no intra-group variation. A few, short axonal projections from the irMCH perikarya were visible in both tadpole and toadlet, but no tracts were seen in either the brain or pituitary.

### 3.3 Discussion

In the adult *Rana temporaria*, immunoreactive MCH cells are located in the postero-lateral hypothalamus, forming an arc-shaped nucleus near the dorsal and ventral infundibular nuclei. Fibres from these cells project to the midbrain and forebrain bundles, but there is no detectable immunoreactivity in either the median eminence or pituitary gland (Baker, 1988).

During development of *R. temporaria*, immunoreactive MCH neurons were first observed in mid-climax tadpoles (TK XXII), in which the MCH perikarya were few and contained sparse irMCH granulation, small nuclei and indistinct nucleoli. This cytology suggests a low level of secretory activity. By TK XXIV, the emergent froglet, MCH perikarya had quadrupled in number, their nuclei were significantly enlarged and irMCH granulation was much more abundant, although fibre tracts were not obvious. These features, suggestive of increased cellular activity, were temporally associated with emergence onto land. In fact, froglets that were unable to leave the water at this stage did not survive. Because the study did not include froglets killed immediately before leaving the water, the results do not reveal whether MCH neuronal activity increased suddenly as a response to emergence onto land, or whether the increase occurred more gradually in preparation for emergence.

The finding that MCH neurons in the permanently aquatic *Xenopus* showed no cytological evidence of increased activity at comparable stages of development strengthens the hypothesis that the infundibular MCH neurons are involved in some way with osmoregulation and water balance. Alternatively, it is possible that for some other reason both *Rana* and *Xenopus* require high levels of MCH at late climax, but that *Xenopus* can achieve this without nuclear hypertrophy due to the higher numbers of MCH cells.

According to another study, in *Xenopus laevis*, MCH immunoreactivity is first observed very early in tadpole life, at pre-metamorphic stage NF 49/50 (equivalent to TK III), with a few neuronal perikarya restricted to the dorsal infundibular nucleus (Korf, H.W; 1990, personal communication). Although the cell bodies are faintly stained, the nerve fibres are more intensely labelled and can be seen to terminate in bouton-like structures in the median eminence and the neurointermediate lobe. By stage NF 54, both the immunoreactivity and the number of cells have increased and fibres are observed in the neurointermediate lobe, pars distalis and PON. By stage NF 61, which is immediately prior to those stages examined in the present work, additional fibres are visible in the lateral hypothalamus, preoptic and pretectal regions.

The present work does not confirm the presence of nerve fibres in either the brain or pituitary of *Xenopus* and, indeed, in adult animals, irMCH staining has not been detected in either the median eminence or pituitary (Baker, 1988). The reason for this discrepancy is not clear since the same antisera (anti-salmonid MCH, Eberle) were used for all three studies.

In the later stages of amphibian metamorphosis, at a time when there is an absolute loss in total body water and in body water expressed as a percentage of dry weight (Brown *et al.*, 1988), there are changes in the secretion and/or synthesis of several hormones with potential osmoregulatory roles, including arginine vasotocin (AVT) and corticosteroids. These hormones or their hypothalamic regulators are thus potential candidates for the neuro-modulatory influence of the MCH system, including mature MCH and other peptides such as NEI, arising from ppMCH.

Arginine vasotocin (AVT) is a neurohypophysial, osmoregulatory hormone which, in amphibians, is produced by magnocellular neurons, located in the anterior pre-optic nucleus (PON) with fibres projecting to the medial, basal and infundibular hypothalamus, median eminence and *pars nervosa*. In some species, for example *Rana catesbeiana*, a few AVT-like perikarya are already detectable in the magnocellular neurons of the PON, suprachiasmatic nucleus (SCN) and hypothalamus as early as stage TK III (Boyd, 1994) but show little activity until TK XII, at which time the cell numbers increase significantly and there is immunoreactivity in the *par nervosa*, indicating the establishment of the hypothalamo-hypophysial tract. The staining in the neural lobe remains intense hereafter but the number of stained irAVT cells in the PON declines after climax (Carr and Norris, 1989). Despite the fact that *Rana catesbeiana* is predominantly, but not exclusively, aquatic one might suppose that the development and activity of a hormonal mechanism for water retention would be different in a terrestrial species, particularly at metamorphic climax, when the animal is preparing to emerge. However, a recent study in the ontogeny of AVT in *Rana catesbeiana* and the highly terrestrial wood frog, *Rana sylvatica* has, surprisingly,

revealed there to be no significant difference in the development of the vasotocinergic pathways, suggesting a common, pre-climactic function (Mathieson, 1996).

When amphibians are exposed to desiccating environments, homeostasis is achieved by increasing the skin permeability to water and by reducing urine excretion, achieved both by reduced glomerular filtration rate and increasing reabsorption from the bladder and kidney. This water balance, or Brunn effect, varies with both taxonomic position and habitat (Jørgensen, 1993). Arginine vasotocin, although it can stimulate all of these responses in some animals, is variable in its effect, for example it does not enhance cutaneous permeability to water in aquatic amphibians, like *Xenopus* (reviewed by Andersen *et al.*, 1992). In a recent and extensive review, Jørgensen (1993) concluded that, despite much evidence on the positive response of AVT release to conditions of salinity, haemorrhage and dehydration, AVT alone does not necessarily play a key role in amphibian water economy. Indeed, dehydration itself is a more potent stimulant to increased water uptake than injections of AVT (Christensen and Jørgensen, 1972).

However, apart from its osmoregulatory functions, AVT has also been shown to stimulate the release of ACTH from the pituitary, in *Rana ridibunda* (Tonon *et al.*, 1986). Immunoreactive AVT is apparent in the bullfrog median eminence and around the portal vessels at TK XVI, suggesting a route of delivery to the anterior pituitary corticotrophs (Carr and Norris, 1989). As in fish and mammals, amphibian ACTH release is also stimulated by CRH (Antoni, 1986) which, in *Rana catesbeiana*, has been shown to reach a peak in the number of stainable neurons at TK XXII (Carr and Norris, 1990). Therefore



two neuronal systems, that are known to activate interrenal steroidogenesis, have developed by mid-climax, at TK XXII, when the median eminence and portal system development is complete (Kikuyama *et al.*, 1993). At this time, there is a dramatic increase in the levels of steroidogenic activity, since in *Bufo bufo*, *Xenopus* and *Rana catesbeiana* corticosterone and aldosterone secretions peak in late metamorphosis, from TK XXII to TK XXIII (NF 62-63). These stages are only one day apart, hence the steroidal surge is very rapid, but then the levels start to decline by the end of metamorphosis (Jaffe, 1981; Jolivet-Jaudet and Leloup-Hatey, 1984; Kikuyama *et al.*, 1986).

Corticosteroids have an osmoregulatory role in amphibians. Both ACTH and glucocorticoids can increase water efflux throughout all the amphibian larval stages (Dodd and Dodd, 1976) for example, compared with a young tadpole, a juvenile bullfrog has over three times less water content per milligram dry weight (Brown and Brown, 1987). Such water loss may be due, in part, to the steroidal surge at mid-climax.

Corticosteroids also play an important part in amphibian metamorphosis, potentiating the response to thyroid hormones (reviewed in Kikuyama *et al.*, 1993). The steroidogenic peak coincides with the maximal levels of thyroxine and triiodothyronine (Jolivet-Jaudet and Leloup-Hatey, 1984) which occur when the amphibian tadpole is undergoing many morphological changes, particularly tail regression, modification of the shape of the head and development of the forelimbs. In addition, in *Xenopus* it has been shown that corticosterone increases the glucose level in body fluid (Hanke and Leist, 1971) and so may

stimulate gluconeogenesis in mid-climax, when the tadpole is not feeding during gut reorganisation (Jolivet-Jaudet and Leloup-Hatey, 1984).

Although there are no data on corticosteroid levels in the *Rana temporaria* tadpole it is probably safe to assume that the corticosteroid surge occurs at the same point in morphological development as for other amphibian species, that is around TK XXII, a time when MCH neurons are first detected in this species. Again, if *Rana* follows the pattern of other amphibians, then the corticosterone and thyroid hormone levels will be declining as the animal emerges from the water, when the MCH neurons show increased activity. It is feasible, therefore, that MCH may contribute toward this post-climactic decline and may achieve this effect indirectly at hypothalamic or pituitary level, as it appears to do in fish (Baker *et al.*, 1985a; Green *et al.*, 1991).

Alternatively, the rise in MCH activity may be concerned solely with the response to the dehydrating conditions that are experienced on emergence. In mammals MCH mRNA is affected by dehydration (Presse and Nahon, 1993) and NEI but not MCH can modify the release of neurohypophysial hormones from the rat neurointermediate lobes *in vitro* (Parkes and Vale, 1993). Since at the end of metamorphosis, AVT levels are lower than at climax (Carr and Norris, 1989), together with the enhanced activity of MCH neurons, it is possible that MCH, or a co-peptide, modifies the release of neurohypophysial hormones. In support of this, a vasopressin analogue (dDAVP) has been shown to stimulate net sodium transport across amphibian skin, an action that can be inhibited by sMCH when it is applied *in vitro*,

suggesting that MCH may act by either modifying the membrane structure or AVP receptor activity and binding capacity (Smriga *et al.*, 1994).

In young rats there is an abrupt increase in the MCH mRNA content at the suckling/weaning transition (Presse *et al.*, 1992) at a time when there is a reorganisation of neural substrates involved in the control of feeding and drinking behaviour. The authors speculated that the rise in irMCH might be related to the central activation of neuronal food and water intake and, since this period is associated with a peak of plasma corticosterone, that glucocorticoids positively regulate MCH mRNA gene activity. Hence weaning in the mammal may parallel emergence in the amphibian, when increased activity of the MCH system is concomitant with dehydration and stress. In contrast to the mammalian pattern, however, in amphibians MCH may lower corticosteroid levels by inhibition of CRH or AVT.

Another feature of emergence for a terrestrial amphibian is that of the change of skin colour which accompanies the change of environment. Whilst cold and wet conditions are associated with melanophore dispersal, heat and desiccation are associated with melanophore contraction (Noble, 1954). Hence the ability to change skin colour relates as much to temperature and humidity as to background colouration. Whilst it has been demonstrated in amphibians that MCH does not cause melanin concentration at the level of the dermal melanophore (Wilkes *et al.*, 1984), as it does in fish (Baker, 1988), that MCH may oppose the release of  $\alpha$ -MSH from the amphibian pituitary has not been reported. The peptide may influence skin colour directly, by inhibiting the release of  $\alpha$ -MSH from the pituitary, or indirectly, by exerting a modulatory effect on  $\alpha$ -MSH-regulating neurons in the

brain. Were this the case, then the increase in MCH activity at emergence may be due to the requirement for a lighter skin colour.

In conclusion, metamorphosis is a time of high hormonal activity. Whilst hypothetical models can be proposed to account for the functional role of an increase in MCH activity in *Rana*, no firm conclusions can be reached. The lack of hyperactivity of *Xenopus* MCH neurons at this time might be due to the considerably higher number of immunoreactive cells present, rather than a genuine species difference to any particular stimulus. Additionally, the increase in cell number and activity in *Rana* may simply be due to normal recruitment as the system develops, for example in the rat, irMCH cells increase in number and granulation during the first week after birth (Baker, 1991). It was not determined whether the changes in nuclear size occurred in non-MCH immunoreactive cells, nor was it possible to delay emergence in order to see if this event could be dissociated from irMCH nuclear changes. Nevertheless, an osmoregulatory role for MCH in the modulation of the dehydration stress experienced by the emerging froglet is proposed. To investigate this matter further, the next chapter includes a study into the response of amphibian MCH to environmental salinity.

## CHAPTER 4

## MCH EXPRESSION IN THE ADULT AMPHIBIAN

## 4.1 Introduction

In the previous chapter it was hypothesised that in amphibians dehydration may be a stimulus for MCH cell activity. In rats, an 8d regime of water withdrawal leads to increased immunoreactive MCH and MCH mRNA (Fellmann *et al.*, 1993) whilst, in contrast, a shorter period of dehydration depresses MCH mRNA in females and in some males, seemingly dependent on genetic strain (Presse and Nahon, 1993). In rats, 2% saline in the drinking water for between 1 and 6 days results in an overall depression of MCH release (Zamir *et al.*, 1986) and synthesis (Fellmann *et al.*, 1993; Nahon *et al.*, 1993) but a few MCH neurons, in the zona incerta and around the fornix and internal capsula, respond to saline challenge by increasing their synthesis of MCH (Presse and Nahon, 1993) suggesting a functional heterogeneity in the MCH cell population. Clearly, osmotic challenge can produce variable results depending on the duration of the stimulus, the genetic strain of the animal and possibly even the gender. It was of interest to determine the reaction of adult amphibian MCH to saline challenge in order to compare with the previous hypothesis relating to dehydration in post-metamorphic amphibians and also to determine if amphibians would follow the mammalian pattern of response or that of teleosts, since in tilapia MCH does not appear to be affected by exposure to salt water (Gröneveld *et al.*, 1995b). The experiments were conducted with immature, home reared *Xenopus laevis* together with immature *Rana temporaria* obtained from a local supplier. The animals were exposed to 35% saline treated water for five or ten days, after which the brains were immunostained for

MCH. Quantitative measurements were made of irMCH cell nuclear areas as a determinant of cell synthetic activity.

Very little is known about MCH in the amphibian, either in respect of its distribution or function. A thorough mapping of peptide in the brain of the marsh frog *Rana ridibunda* (Andersen *et al.*, 1986) produced results that differ from findings in *Rana temporaria* and *Xenopus laevis* (Baker, 1988) in that the principle group of neurons in the marsh frog are located in the pre-optic nucleus (PON) although such cells were not seen in the other two species. Although it would not necessarily be novel to find species differences in hormonal distribution, a previous and unrelated study in this laboratory had revealed the presence of a few MCH cells in the PON in one female *Rana temporaria*. This particular animal had undergone no experimental treatment but was singular in that she carried mature eggs. Since in the saline treatment experiments the focus of interest was on the infundibular region, in which irMCH cells had been previously observed in this species, little attention had been paid to the pre-optic area which, with this one exception, showed no immunoreactive cells. In the rat it has been shown that a group of MCH neurons becomes detectable, by immunocytochemistry and *in-situ* hybridization, exclusively during lactation (Knollema *et al.*, 1992). It was decided, therefore, to thoroughly map MCH cell bodies and axons in both gravid and immature adult *Rana temporaria* using wax-embedded and vibratome sections to see if reproductive activity, as well as salinity, could influence MCH expression in amphibians.

## 4.2 Materials and Methods

### 4.2.1 Amphibians

*Xenopus laevis* tadpoles were obtained from Bristol University and reared to 18 months as outlined in Chapter 2 (section 2.1.3). Adult *Rana temporaria* were obtained from a supplier (Blades Biological Ltd) and maintained as described in Chapter 2 (section 2.1.2).

### 4.2.2 Experiment 1. 35% salinity exposure for 10d in *Xenopus laevis*

In November, five immature (mean weight  $14.5 \pm 2.2$  g) *X. laevis* were transferred to 35% seawater (353 mOsm) and five (mean weight  $17.3 \pm 4.7$  g) were netted briefly and replaced in fresh water. Both groups were sacrificed after 10 d. After anaesthesia in 0.06% (v/v) phenoxyethanol in water, a blood sample was obtained from the hind leg and centrifuged briefly to obtain plasma, used to determine osmotic pressure. The lower jaw and upper palate were removed and the exposed brain perfused with Bouin's fixative before removal. After overnight post-fixation, the tissue was dehydrated and embedded in wax blocks. Serial sections were cut at 5  $\mu$ m and immunostained for MCH using the triple antibody method (Sternberger, 1974) outlined in Chapter 2 (section 2.4.1). Sections were incubated overnight, at room temperature, with anti-rat MCH (837) primary antiserum at x1000 dilution. Following incubation with secondary and PAP antisera, the MCH antibody-antigen complexes were visualised by soaking the sections in freshly prepared DAB solution, with added cobalt chloride (0.03% w/v) for colour enhancement. The sections were then counterstained in eosin, cleared and mounted.

For each animal, camera lucida drawings were made, under oil immersion, of x150 irMCH cell nuclei from the dorsal infundibular (NID) region. The cross sectional surface area of each cell nucleus was calculated using the maximum and minimum diameters ( $\pi \times d^1/2 \times d^2/2$ ).

#### **4.2.3 Experiment 2. 35% salinity exposure for 5d in *Rana temporaria***

In June, four immature *R. temporaria* (mean weight  $18.2 \pm 1.1$  g) were placed in a tank, identical to that of the control tank, but with 1 cm 35% salt water in the base (351 mOsm). Four control animals (mean weight  $20.1 \pm 2$  g) were briefly handled and replaced in the fresh water tank. After 5d both groups were sacrificed and brains fixed by aortic perfusion (Chapter 2 section 2.3.2) of heparinised buffer, followed by approximately 50 ml Bouin's fixative. Following overnight postfixation, dehydration and wax embedding, transverse sections were cut at 5  $\mu$ m and immunostained for MCH using anti-rMCH (837) antiserum at x1000 dilution. After visualisation with DAB solution sections were counterstained with haematoxylin but colour enhancement with cobalt chloride was not employed due to the presence of melanin deposits in the *Rana* brain. Initially, x150 nuclear measurements were made from each brain, as with *Xenopus laevis*. Data were analysed by two way ANOVA.

Subsequently, the total number of irMCH cells per brain were counted in male animals (n=3 in each group) and all the irMCH cell nuclei were measured. The number of cells and nuclear surface areas were plotted as 50  $\mu$ m tranches (ie. in 10 x 5  $\mu$ m sections) along the anterior to posterior axis of the brain. Corresponding segments in different animals were analysed using two way ANOVA.



#### **4.2.4 Experiment 3. 35% salinity exposure for 5 d in male *Rana temporaria***

Experiment 2 was repeated in July, using the same protocol, with immature (mean weight  $19 \pm 0.9$  g) male frogs (n=5 in each group). Both control and experimental animals were sacrificed after 5d. Blood samples were obtained from the hind leg, before perfusion, and the plasma used to determine osmotic pressure. Nuclear measurements were made from all irMCH cells in each brain. The total cell numbers and nuclear measurements were, again, determined for 50  $\mu$ m tranches and plotted along the anterior to posterior axis of the brain. Corresponding segments in different animals were analysed using two way ANOVA.

#### **4.2.5 Experiment 4. Mapping MCH neurons and fibres in adult *Rana temporaria***

Two immature, adult female frogs were used, in October, for a complete mapping of MCH neurons and fibres in the brain. The animals were anaesthetised in 0.06% (v/v) phenoxyethanol and perfused, via the aorta, with heparinised PBS (0.01M pH 7.4) and then approximately 50 ml Bouin's fluid fixative. The brains were removed and postfixed overnight before wax embedding. Serial transverse or sagittal sections were cut at 5  $\mu$ m and immunostained (section 2.4.1) using antisera against rat MCH (837) and salmonid MCH (Eberle) at x1000 dilution. Both antisera gave identical results. The specificity of these antisera was confirmed by incubation of alternate sections with pre-adsorbed antiserum (10  $\mu$ g MCH in 500  $\mu$ l diluted primary antiserum) or normal antiserum. Camera lucida drawings were made of complete brain sections to show the location of irMCH neurons and fibre tracts.

#### **4.2.6 Experiment 5. Investigating irMCH distribution in the reproductively mature *Rana temporaria* using vibratome sections**

Three mature female *Rana*, carrying eggs, were obtained from a commercial supplier in late April. One frog was sacrificed immediately on delivery whilst the remaining animals were housed in the aquarium for a further 3 d before being killed. During this time both frogs had shed their eggs into the tank. Brain tissue was fixed as described in the preceding sections, following perfusion of Bouin's fluid. Vibratome sections were cut at 100  $\mu$ m and immunostained for MCH, by the streptavidin-biotin method (Chapter 2, section 2.4.2), using anti-rat MCH (837) primary antiserum at x2000 dilution.

This investigation was extended in mid-June when four mature females and four mature males were obtained from the same supplier. It was determined from the supplier that these animals had been breeding two weeks previously but at *post mortem* it was clear that the females did not carry eggs and the male testis weight was low (mean weight  $0.04 \pm 0.001$  g). The brains were fixed in Bouin's fluid, as before, and vibratome sections were cut at 100  $\mu$ m in either sagittal, transverse or horizontal planes. In two brains (x1 female, x1 male) sections were cut at 50  $\mu$ m and alternately immunostained for MCH and  $\alpha$ -MSH using the streptavidin-biotin method with anti-salmonid  $\alpha$ -MSH (Bowley) and anti-rat MCH (837) primary antisera, both at x5000 dilution.

### **4.3 Results**

#### **4.3.1 The effect of exposure to 35% salinity in *Xenopus laevis***

The weight, sex, osmotic pressure and mean irMCH nuclear areas, after 10 d exposure to 35% salinity, are shown in Table 3. In this first experiment, because gender could not be

determined before autopsy, the sex ratio is different in control and experimental groups.

One control animal died from unknown causes.

**Table 3**

**Weight, sex, plasma osmotic pressure and mean irMCH cell nuclear surface areas in *Xenopus laevis* after 10 d exposure to 35% salinity.**

Sample	Sex	Weight (g)	Osmotic pressure (mOsm kg <sup>-1</sup> )	Mean (±SEM) MCH nuclear area (µm <sup>2</sup> ) n=150 cells
Control	M	12.08	290	43.5 ± 0.6
	M	14.96	257	48.1 ± 0.7 <b>45.8 ± 0.5 (♂)</b>
	F	16.43	201	38.9 ± 0.6
	F	16.71 <b>14.5 ± 1</b>	300 <b>262 ± 22</b>	38.7 ± 0.5 <b>38.8 ± 0.3 (♀)</b>
Saline	M	12.97	361	46.8 ± 0.9
	M	19.06	358	45.0 ± 0.6
	M	15.14	400	46.9 ± 0.7
	M	25.52	362	44.4 ± 0.7
	M	22.40 <b>22.4 ± 2.3</b>	367 <b>370 ± 8</b>	47.9 ± 0.9 <b>46.2 ± 0.6</b>

NB. Highlighted figures represent mean ± SEM for each group. Plasma osmotic pressures were significantly different between control and experimental groups ( $P < 0.001$ ). Female MCH nuclear areas were lower than male controls (not tested) but nuclear areas of control and saline treated males were not significantly different from one another.

The mean plasma osmotic pressure rose from  $262 \pm 22$  mOsmkg<sup>-1</sup> in controls to  $370 \pm 8$  mOsmkg<sup>-1</sup> in saline-treated toads. The osmolarity of the water in the saline treatment tank was measured at 353 mOsmkg<sup>-1</sup>. The irMCH nuclear areas in saline-treated males (n=5) were not significantly different from each other or from the control males (n=2). Nuclear areas in the two control females were not significantly different from one another but, although not tested, appeared to be lower than those in either the control or saline-treated males. There was no statistical relationship either between irMCH nuclear surface areas or

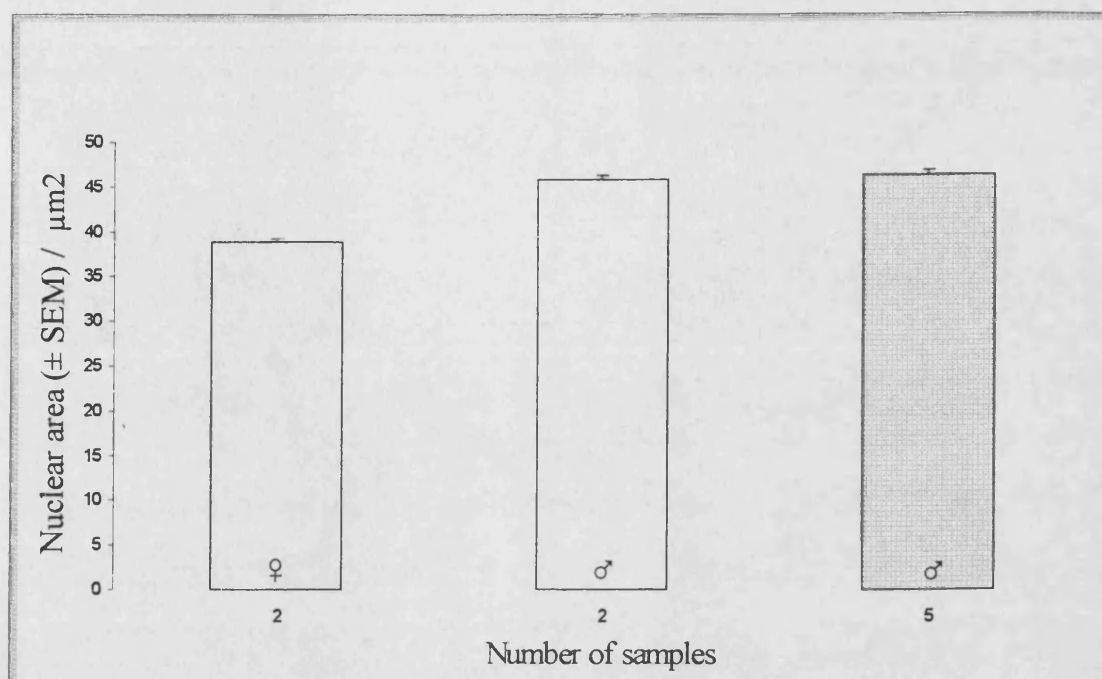


Fig 5 Histogram to show mean ( $\pm$  SEM) irMCH cell nuclear surface areas in *Xenopus laevis* after exposure to 35% salinity for 10 days. The shaded column refer to the saline-treated group. 150 nuclei were measured in each animal, taken from the NID population.

plasma osmotic pressure. Figure 5 shows the irMCH nuclear surface areas for *X. laevis* after 10 d exposure to 35% salinity.

This pilot experiment with *Xenopus laevis* was ultimately unsatisfactory since the unequal sex ratio and the apparent difference in MCH nuclear areas between males and females rendered the sample size inadequate for significant statistical analysis. However, the results suggest that exposure to 35% salinity has no effect on MCH cells in male *Xenopus laevis* and that the nuclear surface areas of irMCH cells are smaller in female than male toads.

### 4.3.2 The effect of exposure to 35% salinity in *Rana temporaria*

In experiment 2, again, due to their immaturity, the gender of these animals could not be determined until *post mortem*. The weight, sex and irMCH nuclear areas are shown below in Table 4 and the latter are also summarised in Figure 6.

**Table 4**  
**Weights, sex and mean irMCH cell nuclear surface areas**  
**in *Rana temporaria* after 5d exposure to 35% salinity**

Sample	Sex	Weight / g	Mean ( $\pm$ SEM) MCH nuclear area ( $\mu\text{m}^2$ ) n=150 cells
Control	F	17.13	33.3 $\pm$ 0.5
	M	18.02	38.5 $\pm$ 0.5
	M	19.73	38.5 $\pm$ 0.6
	M	17.99 <b>18.2 <math>\pm</math> 0.5</b>	41.6 $\pm$ 0.6 <b>39.5 <math>\pm</math> 0.5 (<math>\sigma</math>)</b>
Saline	F	18.53	39.6 $\pm$ 0.8
	M	21.12	48.3 $\pm$ 1.1
	M	22.50	44.9 $\pm$ 0.7
	M	18.41 <b>20.1 <math>\pm</math> 1</b>	47.3 $\pm$ 0.8 <b>46.8 <math>\pm</math> 0.9 (<math>\sigma</math>)</b>

NB. Highlighted figures represent mean  $\pm$  SEM for each group. The mean MCH nuclear area of the single control female was smaller than that of the single saline treated female and from both male groups (not tested). The nuclear areas of saline treated males were significantly higher than those of control males ( $P < 0.001$ ).

Because the frogs were immature, gender could not be determined before autopsy. The apparent sexual dimorphism in irMCH nuclear sizes precluded combining the data for males and females and hence only the sample size for male animals was adequate for statistical analysis. Although the small sample numbers precluded statistical analysis, it appeared that

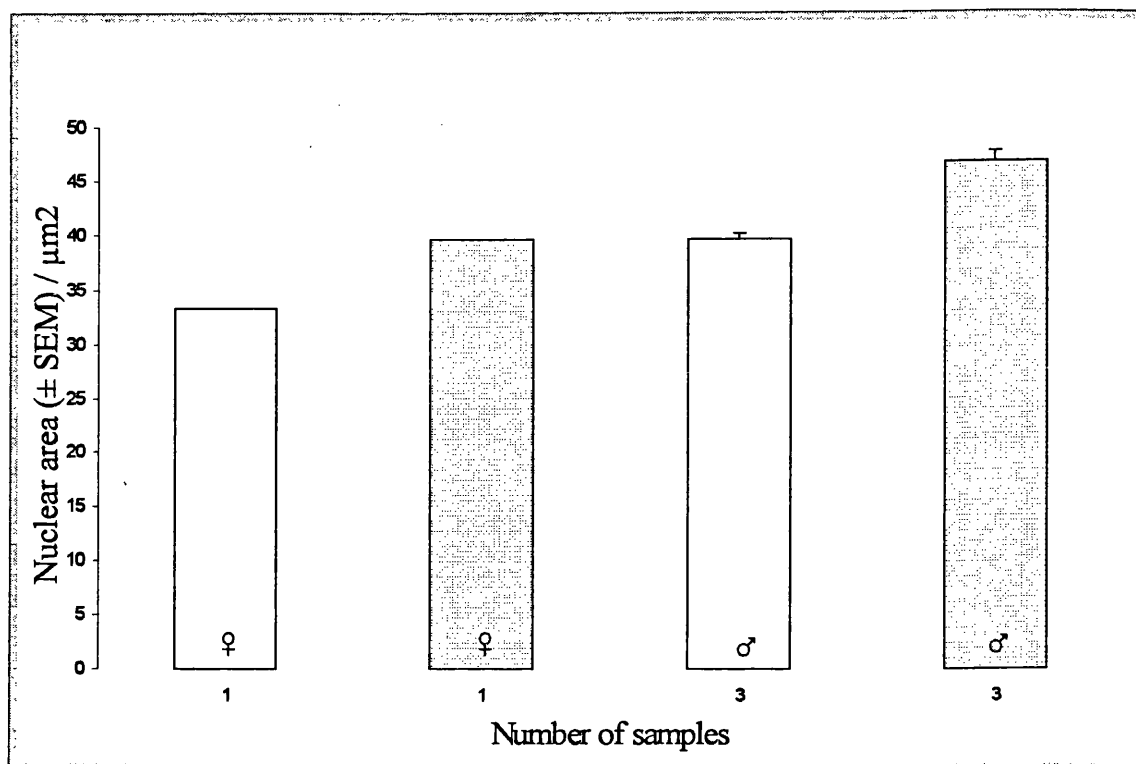


Fig 6 Histogram to show mean irMCH cell nuclear areas in *Rana temporaria* after exposure to 35% salinity for 5 days. The shaded columns represent saline-treated groups. 150 nuclei were measured in each animal, taken from the NID population.

the irMCH nuclear areas in female animals were smaller than those in males, as observed previously.

In males, a five day exposure to 35% saline resulted in a significant increase in MCH nuclear area ( $P < 0.001$ ). Cytoplasmic granulation was increased in the saline-treated animals and axonal tracts were also more obvious, extending into many brain areas including the olfactory lobes, median eminence and optic tecta. When all the irMCH nuclear areas were counted and the number plotted along the anterior to posterior axis (Figure 7), it became clear that most of the cells in the NID region showed increased nuclear activity in response to saline.

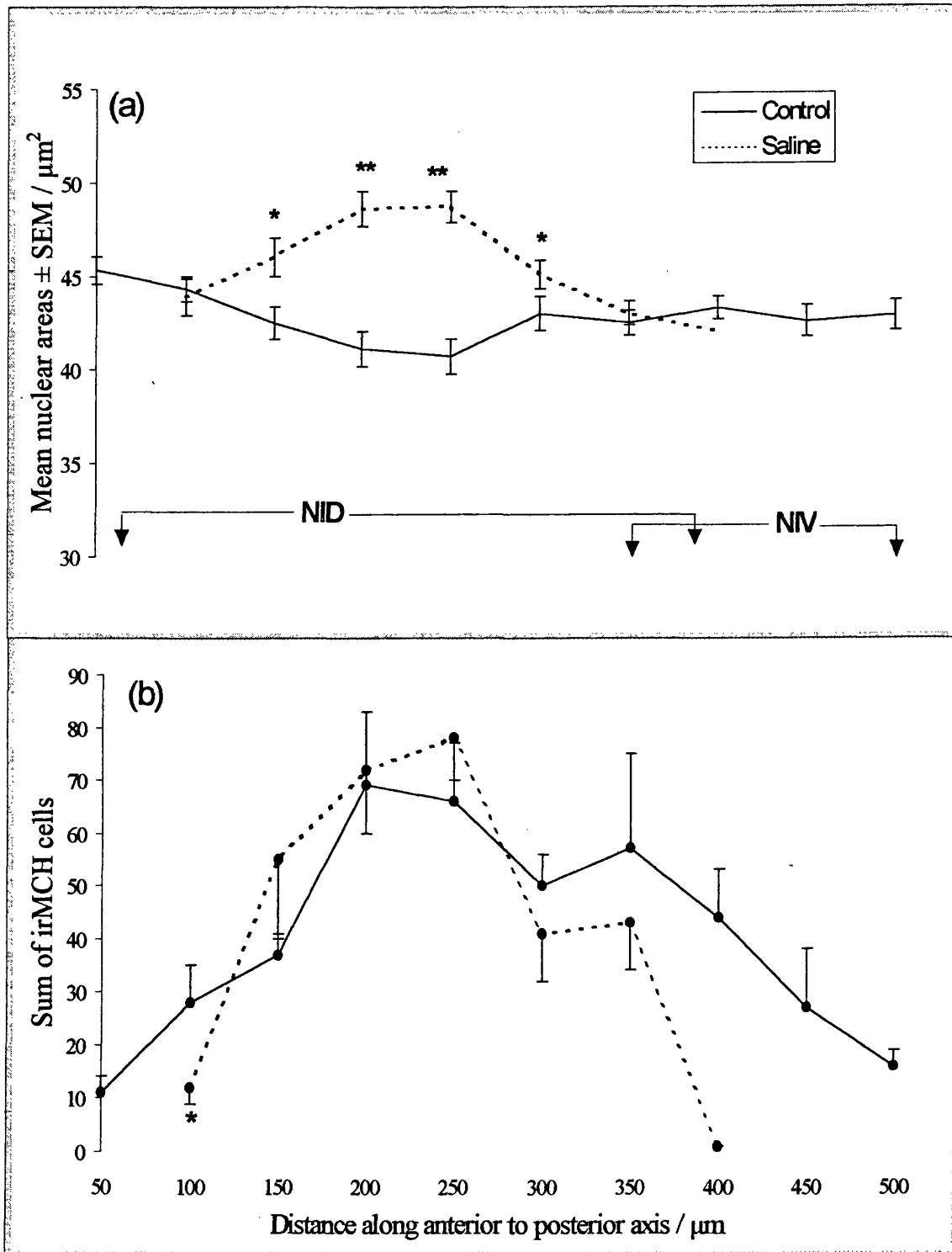


Fig 7 Effect of 5 d exposure to 35% salinity on male *Rana temporaria* (n=3) (a) Mean ( $\pm$ SEM) irMCH cell nuclear areas of NID and NIV neurons. (b) Mean ( $\pm$ SEM) number of irMCH cells in NID and NIV. NB X-axis labels apply to both graphs. \* P < 0.01 and \*\* P < 0.001

The number of irMCH cells in the control and experimental groups did not differ markedly along the axis, except that cells were not seen in the most anterior region of the NID, nor in the NIV, after saline treatment. It is possible that this was due to degranulation in these regions.

Analysis of Experiment 3 showed essentially the same results (Table 5; Figure 8). Again, the plasma osmotic pressures of saline treated frogs were significantly higher than those of control animals ( $P < 0.001$ ). The salinity of the experimental tank was measured at 351 mOsmkg<sup>-1</sup>.

Table 5

## Weight and plasma osmotic pressures in male

*Rana temporaria* after 5d exposure to 35% salinity.

Sample	Weight / g	Osmotic pressure / mOsmkg-1
Control	12.8	251
	18.3	250
	20.4	290
	16.0	251
	17.2 <b>18.4 ± 1.3</b>	254 <b>259 ± 8</b>
Saline	17.6	455
	16.6	376
	18.2	380
	16.9	399
	17.1 <b>17.9 ± 1.1</b>	323 <b>386.6 ± 21</b>

NB Highlighted figures are the mean ( $\pm$  SEM) for each group. The mean plasma osmotic pressure of saline treated male frogs was significantly higher than that of controls ( $P < 0.001$ ).



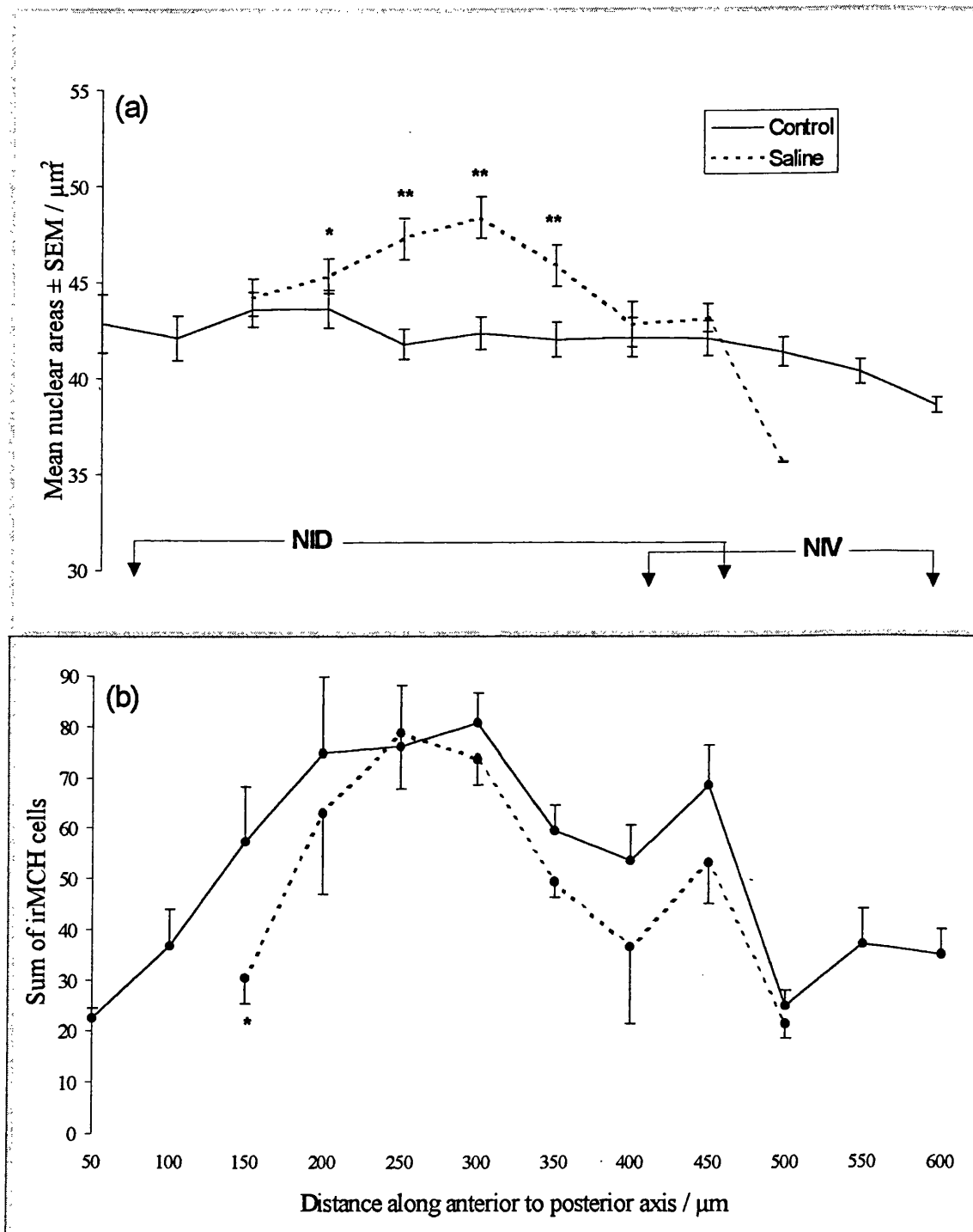


Fig 8 Effect of 5 d exposure to 35% salinity in male *Rana temporaria* (n=5) (a) Mean (±SEM) irMCH cell nuclear surface areas of NID and NIV neurons. (b) Mean (±SEM) number of irMCH cells in NID and NIVirMCH cells. NB. X-axis labels apply to both graphs. \*  $P < 0.01$  and \*\*  $P < 0.001$ .

Although irMCH cells in saline-treated frogs were slightly less abundant than controls in this experiment the NID cells showed, once again, a significant increase in nuclear area. Compared with controls (Figure 9a), saline-treated irMCH cells had increased cytoplasmic granulation, darker and more rounded nuclei, prominent nucleoli and immunoreactive fibre tracts were more marked (Figure 9b). The NIV and more anterior NID neurons were, again, not visible.

In summary, the results suggest that male frogs subjected to raised environmental salinity, in which the plasma osmotic pressure increases, show increased granulation of irMCH cytoplasm, prominent fibre tracts, enlarged cell nuclei and darker nucleoli, features consistent with enhanced cellular activity. Based on Experiment 2, there is slight evidence to suggest that female frogs show a similar response to salinity and that both control and saline-treated irMCH cell nuclei were smaller than in male animals.

#### **4.3.3 Mapping MCH cell bodies and neuronal tracts in adult *Rana temporaria* using wax embedded sections**

Drawings of transverse brain sections are shown in Figure 10. In the adult frog, as in the tadpole, there appeared to be two confluent populations of MCH perikarya located in the posterior hypothalamus. A few small, lightly stained cells were present posteriorly in the region of the NIV, close to the infundibular recess. The majority of immunoreactive irMCH cells were larger, with more densely stained nuclei and small, dark nucleoli. This cell group was located in an area spanning the NIV and NID to a point above the optic chiasma.

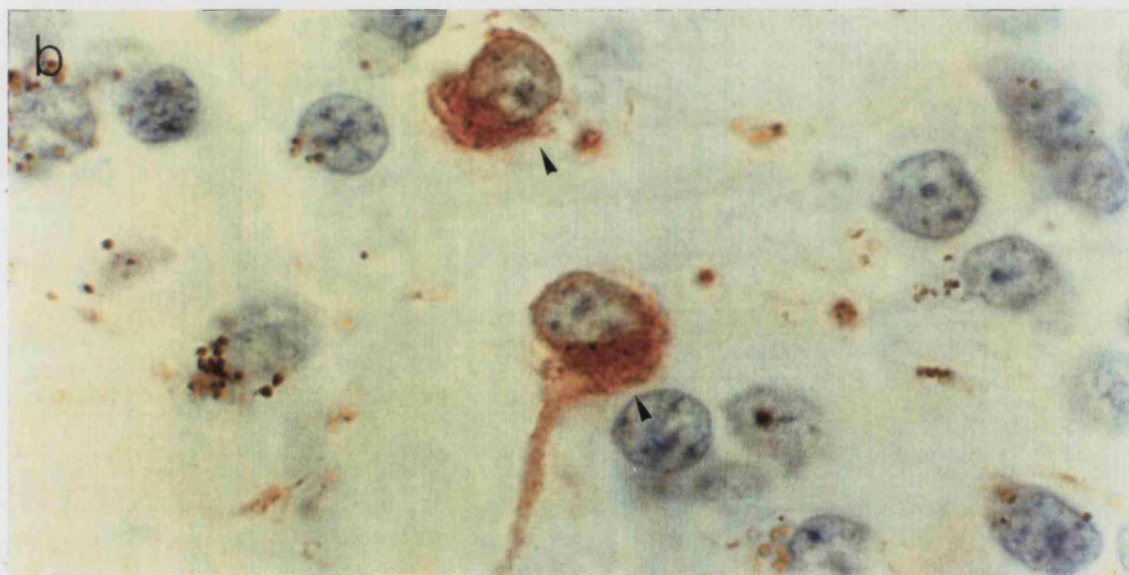
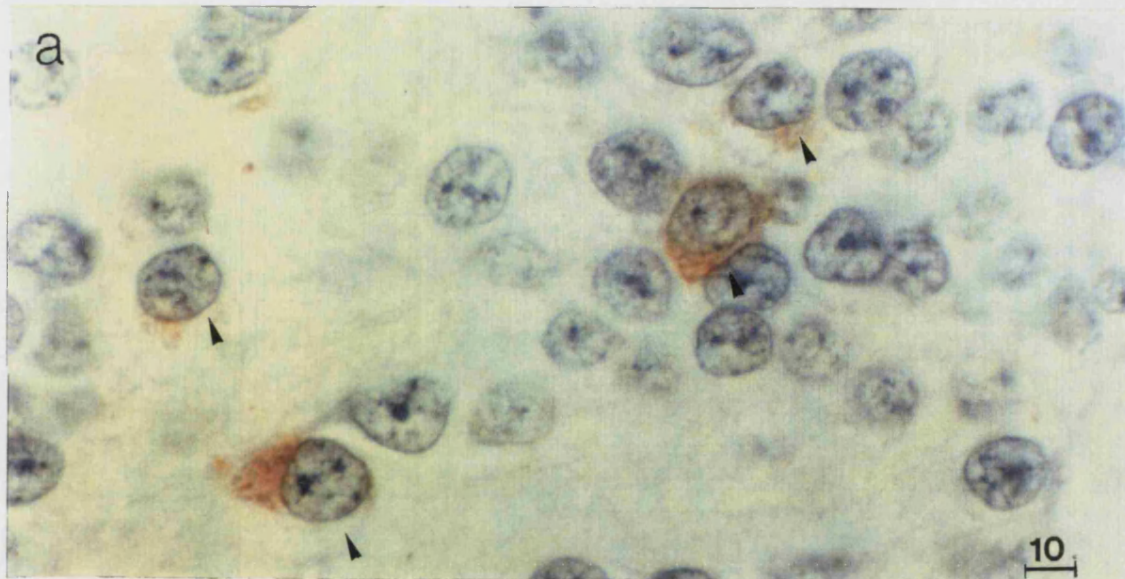


Fig 9. Immunoreactive NID MCH neurons in control and saline treated male *Rana temporaria*. (a) irMCH neurons (arrowed) in control frog showing small nuclei and palely staining cytoplasm. (b) irMCH neurons (arrowed) in NID region of frog exposed to 35% saline for 5 days. The nuclei are significantly ( $P < 0.001$ ) larger than those in control animals and there is increased granularity in the cytoplasm, together with an increased number of immunoreactive fibres throughout most regions of the brain. Scale bar represents  $\mu\text{m}$ .

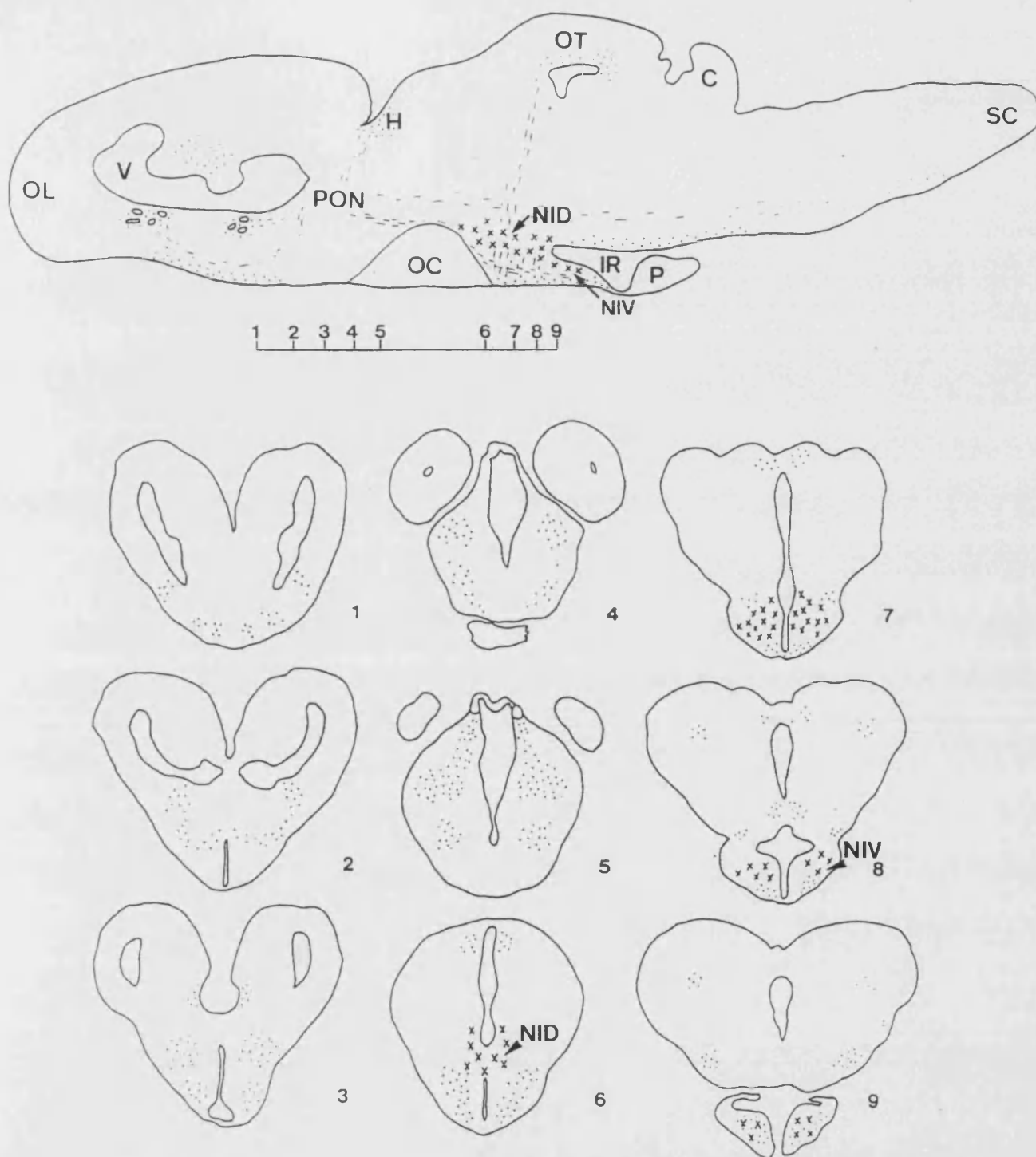


Fig 10. Camera lucida drawings of sagittal and transverse sections of *Rana temporaria* brain. The irMCH cell bodies are marked (x) and neuronal tracts are represented by dashed lines or dots. Transverse sections are numbered according to their position on the scale shown against the sagittal section. OL olfactory lobe, PON preoptic nucleus, H habenular nucleus, OC optic chiasma, OT optic tectum, IR infundibular recess, NIV ventral infundibular nucleus, NID dorsal infundibular nucleus, C cerebellum, P pituitary gland, SC spinal cord.

By superimposing camera lucida drawings from several serial sagittal sections onto one schematic section, it was apparent that major irMCH neuronal tracts made connections with two regions in the olfactory lobe, with the habenular nucleus, the optic tectum, median eminence and, to a lesser extent, the spinal cord. Both tracts to the olfactory lobe terminated near the first/second ventricles. Although cell bodies were seen only in the posterior hypothalamus, with none in the preoptic region, many tracts were visible in the preoptic area, apparently associated with unstained cells in this region. No immunoreactivity was observed in the pituitary gland.

Alternate slides of sections from an adult frog were used to check the specificity of the anti-sMCH and anti-rMCH antisera by immunosorption. No immunoreactive cells were visible on those sections in which antiserum had been pre-incubated with MCH.

#### **4.3.4 Investigating irMCH distribution in the reproductively mature *Rana temporaria* using vibratome sections**

Thick (100  $\mu$ m) vibratome sections revealed more details of MCH cell shape and fibre distribution than the thinner wax sections. Seven sexually mature females were examined, one carrying eggs, two that had shed their eggs in the previous two days, and four that had been reproductively active two weeks previously. In all animals, irMCH perikarya were located in the dorsal and, to a lesser extent, ventral infundibular nuclei confirming previous observations in wax-embedded sections. Whilst the majority of the neurons appeared to be monopolar, there were clearly also a few bipolar cells present. The tracts from these cells coursed ventro-laterally, joining a thick network of fibres on the ventral floor of the

infundibulum which extended into the external zone of the median eminence but were not apparent in any part of the pituitary gland (Figure 11).

In the egg-carrying female, in the anterior pre-optic nucleus (APON) there was a group of irMCH neurons arranged in linear fashion, neighbouring the ependymal layer bordering the third ventricle (Figure 12). Cells of this group were fewer in number than the NID neurons and less intensely stained but appeared to be of about the same size, with axonal projections coursing laterally. The entire preoptic region was heavily immunostained with nerve fibres but it was not possible to say whether such fibres arose solely from these PON cells. In one spent female the PON group of irMCH neurons was also visible but consisted of only a few cells whilst in the other spent female, the four post-reproductive females and four post-reproductive males the PON cells were not stained.

In the egg-carrying female and the two spent females only, a third group of irMCH neurons was identified in the lateral septal nucleus (NLS). These cells were concentrated in a small area as viewed in transverse section (Figure 13) and in the two spent females consisted of only about a dozen cells but, in the egg-carrying female, appeared on consecutive sections spanning at least 500  $\mu\text{m}$  along the anterior-posterior axis and contained in excess of three-hundred cells. The perikarya were smaller but more intensely stained than either the PON or NID cells. The axons from these neurons were not easy to trace since there was considerable fibre staining in this region which crossed both the medial septal nucleus and medial pallium.



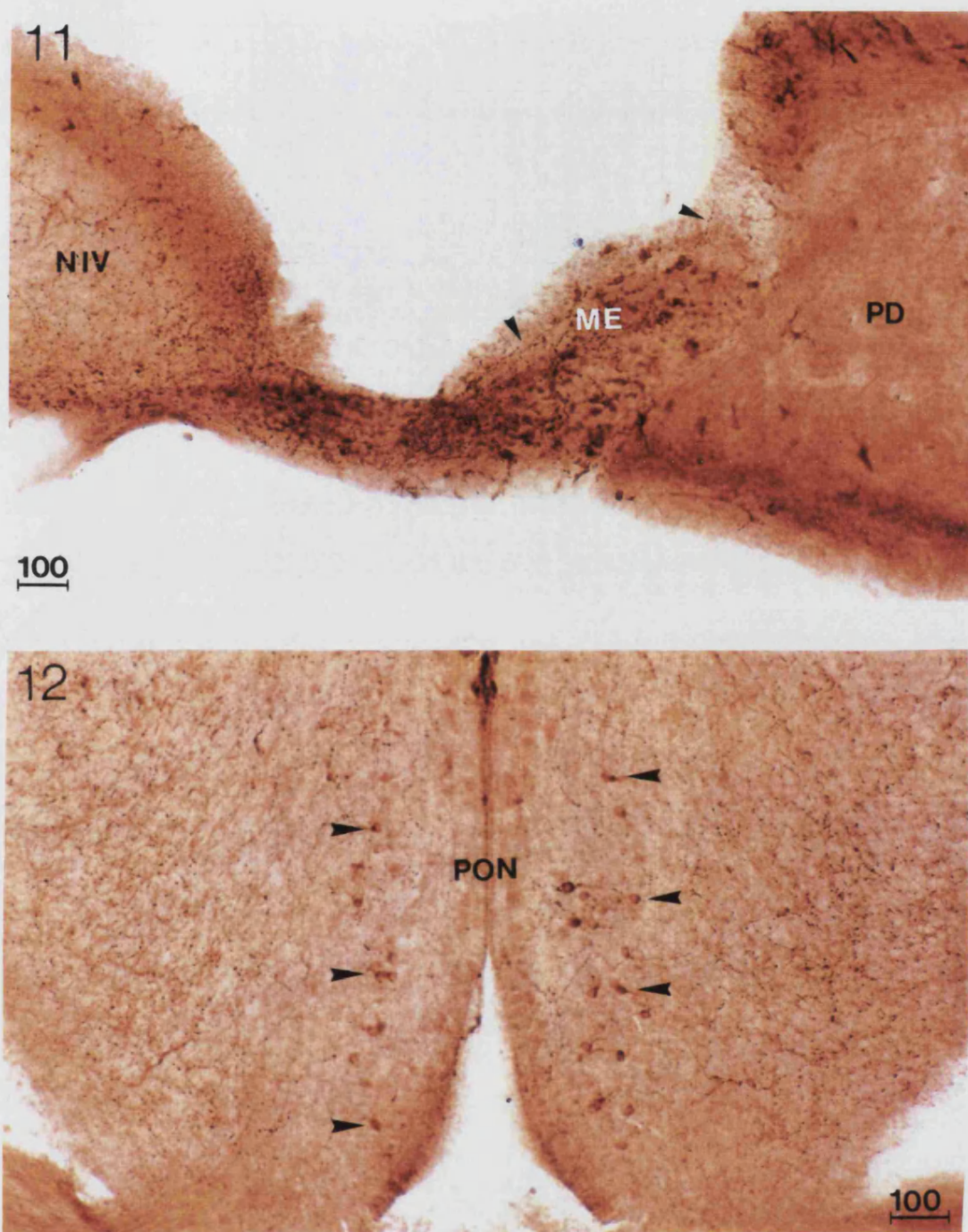


Fig 11. Sagittal vibratome section of adult female *Rana temporaria* to show irMCH fibres (arrowed) in the NIV region and median eminence, but not in the pituitary gland. NIV ventral infundibular nucleus, PD pars distalis, ME median eminence. Scale bar represents  $\mu\text{m}$

Fig 12. Transverse vibratome section of egg-carrying female *Rana temporaria* to show irMCH neurons (arrowed) in the preoptic nucleus (PON) of the anterior hypothalamus. Although there are many immunoreactive fibres visible in the area, direct connections with these cells are not established. These neurons were not stainable in immature animals but a few cells were still visible in those frogs who had recently shed eggs. Scale bar represents  $\mu\text{m}$

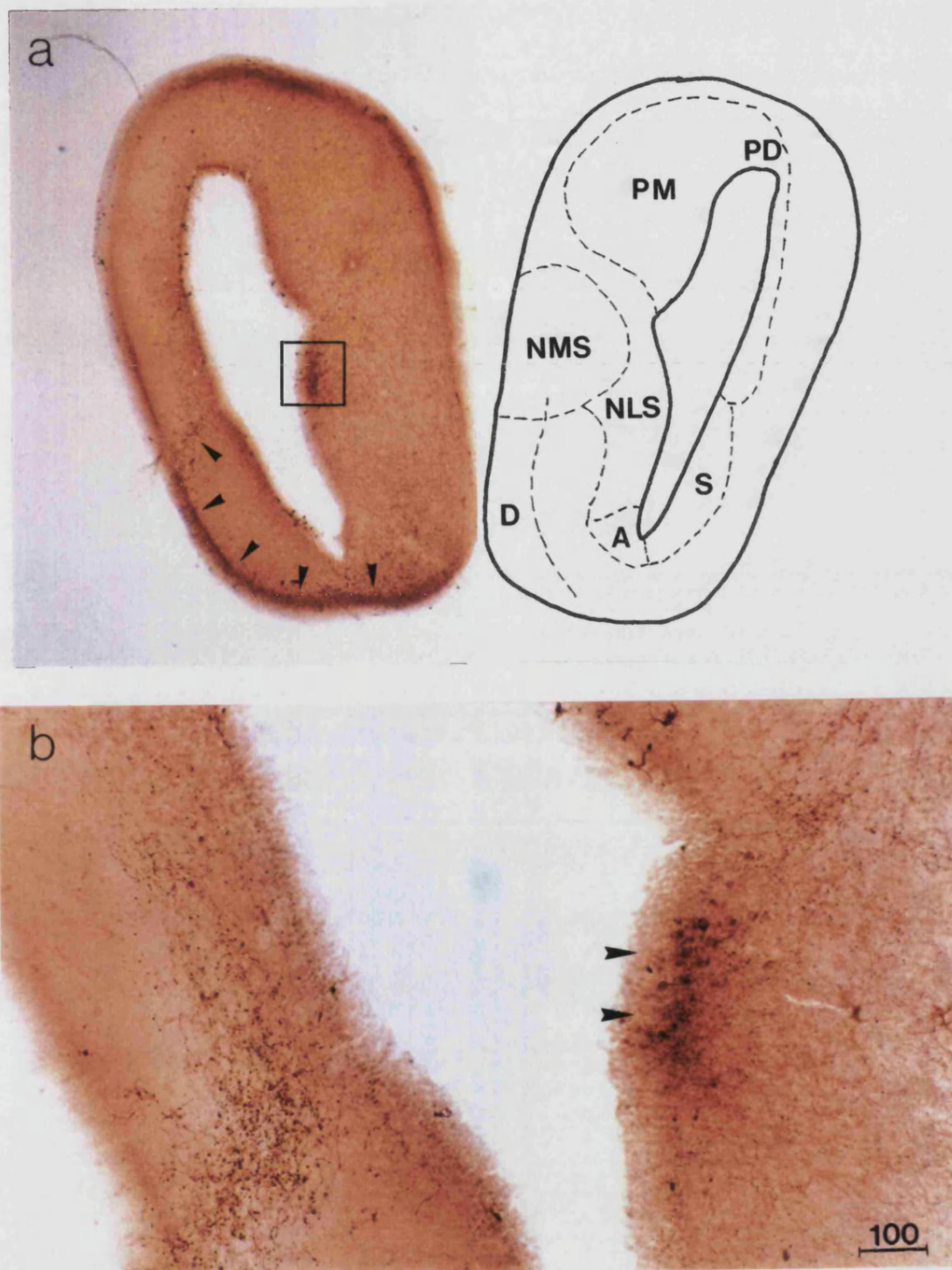


Fig 13. Transverse vibratome sections through the telencephalon of egg-carrying female *Rana temporaria*. (a) Left :irMCH cell bodies (within square) located in the lateral septal nucleus with a heavily stained nerve bundle (arrowed) . These fibres may originate from the NLS groups of irMCH cells but this cannot be seen in transverse section. Right : Drawing to show brain regions NMS medial septal nucleus, NLS lateral septal nucleus, D nucleus of the diagonal band of Broca, A nucleus accumbens septi, S striatum, PD dorsal pallium, PM medial pallium. The drawing is labelled according to Wada *et al* (1980) (b) Enlargement of figure 4(a) showing irMCH cell bodies and fibres. Scale bar represents  $\mu\text{m}$ .



A major fibre tract extended ventrolaterally from the NLS irMCH group and may have been continuous with a tract on the far side of the first/second ventricle but a connection between these cells and fibres could not be established in transverse section.

In one male and female frog, alternate sections were immunostained for  $\alpha$ -MSH. The immunoreactive  $\alpha$ -MSH cell bodies were located in the most ventral aspect of the NIV with nerve fibres extending both ventrally towards the median eminence and dorsally towards the NID. There was intense labelling in the intermediate lobe of the pituitary and fibres were also seen in the ventral telencephalon and along the ventral floor of the hypothalamus as previously reported in *Rana ridibunda* (Benyamina *et al.*, 1986).

#### 4.4 Discussion

These studies have addressed two problems: The first part seeks to determine if a hyperosmotic challenge affects MCH neurons in the posterior hypothalamus. The second part investigates why initial immunocytochemical studies on *Rana temporaria* have revealed only the infundibular MCH neurons whereas anterior hypothalamic MCH neurons have been described in the marsh frog *Rana ridibunda* (Andersen *et al.*, 1986).

In the first two saline experiments there were insufficient numbers of females, either of *Xenopus laevis* or *R. temporaria*, to allow any firm conclusions regarding the response of MCH neurons to osmotic challenge. However, one interesting observation is that in both species the irMCH cell nuclei were consistently smaller in females than males. A gender difference in cellular size is not unique, for example irAVT cells in the supra-chiasmatic

nucleus of female bullfrogs are larger than the male equivalents (Boyd *et al.*, 1992). This confirms that certain areas of the brain are sexually dimorphic and that hormone synthesis or release by cells in those loci may be regulated in part by sex steroids. Whether there are such receptors on MCH cells has not been established.

In *R. temporaria* males, exposure to 35% saline for 5 d resulted in significant increases in irMCH nuclear cross sectional areas together with increased granularity, both of the cell cytoplasm and fibres. Increased granularity alone might indicate a inhibition of peptide release but together with increased nuclear size and prominent nucleoli suggests enhanced synthetic activity. These findings parallel those in the newly metamorphosed froglet emerging onto land, an event accompanied by increased MCH neuronal activity. Again, there are two possible interpretations, that the peptide is produced in response to osmoregulatory stimuli or as a result of non-specific stress accompanying the change in environment.

Although the sample numbers of *Xenopus laevis* were low it appears that a 10 d exposure to 35% salinity had no significant effect on irMCH synthetic activity in male toads, although they may have adapted within this time period. The survival of anuran amphibians in hyperosmotic environments depends on the adjustment of renal/glomerular mechanisms, changes in skin and bladder permeability and the transport of water and solutes in order to conserve osmotically free water. In some amphibians, an adaptation to hyperosmolarity involves a switch from ammonotelic to urotelic waste elimination, such that urea is conserved and raises the internal plasma osmolarity without affecting electrolyte content

(Shpun *et al.*, 1992). Such a mechanism allows such animals to adapt to very hyperosmotic conditions, for example *Bufo viridis* can acclimate in salt or urea solutions of up to 800 mOsm (Shpun *et al.*, 1993). Whilst *Xenopus laevis* employs this system (Bolton and Henderson, 1987) *Rana temporaria* does not (Ireland, 1973) and cannot tolerate hyperosmotic solutions much above 350 mOsm. Since amphibians maintain their internal osmolarity approximately 10% above that of the external medium (Hanke and Kloas, 1994), a theoretical maximum plasma osmolarity for this species is about 385 mOsm, which closely accords to the mean plasma osmotic pressures for the saline-treated group ( $386 \pm 21$  mOsmkg<sup>-1</sup>) in the all-male saline experiment in *Rana*.

It would appear, therefore, that these frogs were at the upper limit of their salinity tolerance during the course of these experiments and it is probable that such exposure was physiologically stressful. Since MCH has been shown to modulate the stress response in fish (Baker *et al.*, 1985a; Baker *et al.*, 1986; Green *et al.*, 1991) and mammals (Nahon *et al.*, 1989; Jezova *et al.*, 1992; Bluet-Pajot *et al.*, 1995) it is probable that this may also be true in amphibians. This being the case it is difficult to divorce the stress response from the osmotic response in the interpretation of the observed increase in MCH cellular activity in *Rana*. Since *Xenopus* exhibits a higher tolerance to a hyperosmotic environment, due to the raising of internal urea levels (Bolton and Henderson, 1987), it is feasible that neither an osmotic nor a physiological stress was imposed which may account for the failure of MCH neurons to show any significant response in this species.

In an osmoregulatory role, MCH may influence the synthesis or release of the principle amphibian neurohypophysial hormone, arginine vasotocin (AVT). In anurans,

vasotocinergic neurons are located in the anterior PON with fibres to numerous regions of the brain including the pallium, optic tectum, thalamus, median eminence and neural lobe of the pituitary (reviewed in Andersen *et al.*, 1992). This hormone is a potent vasoconstrictor that reduces the renal glomerular filtration rate, hence urine flow, and acts as a secretagogue of corticosterone and aldosterone. These steroids increase sodium transport across the epithelial surfaces of the skin and bladder and mobilise energy reserves by raising blood glucose levels during stress.

Historically, AVT has also been thought responsible for increasing skin permeability to water and reducing urine output in terrestrial amphibians (reviewed in Hanke and Kloas, 1994). However, a peripheral vasoconstrictor such as AVT is unlikely to contribute to skin permeability by increasing cutaneous blood flow and indeed AVT has been demonstrated to decrease such blood flow even when skin permeability is enhanced (Malvin, 1993). Rather, the increase in permeability is thought to result from the insertion of water channels in the skin and recently it has been shown that such channels, termed aquaporins, are markedly increased in response to salt acclimation in both the skin and bladder of some amphibians (Abrami *et al.*, 1995). It may be, therefore, that antidiuretic hormones influence the synthesis of aquaporins in terrestrial amphibians.

Fully aquatic amphibians do not show a strong response to neurohypophysial hormones, since AVT neither increases the skin permeability to water nor reduces the urine outflow in *Xenopus* (Bentley, 1982). Such a mechanism, necessary for a terrestrial amphibian to counteract dehydration, would presumably, in an animal surrounded by water, lead to fatal hyperhydration. It seems logical that the hormonal responses to salinity or dehydration must

therefore depend on the environment which the frog or toad inhabits. In the current experiments exposure to a hypertonic medium produced contrasting reactions between terrestrial *Rana* and aquatic *Xenopus* in the synthetic activity of irMCH neurons. Since *Rana* is more likely than *Xenopus* to experience desiccation, MCH, like AVT, may exert osmoregulatory effects inappropriate to the aquatic species.

Studies into the circumstances under which AVT is released have yielded variable results but it is believed that the principle stimulus is hypovolaemia caused by haemorrhage or dehydration (reviewed in Jørgensen, 1993). In *Rana ridibunda*, water deprivation for 3 h leads to a threefold increase in serum AVT whilst saline injections have no effect even when the plasma osmolarity is the same in both experiments (Nouwen and Kühn, 1985). Whilst high plasma osmolarity may not in itself be a stimulus for AVT release, a five day regime of salt loading in a terrestrial amphibian, such as experienced by *Rana* in the current experiments, is likely to have caused dehydration and hence activity of the AVT system.

A relationship between mammalian AVP and the MCH system has been investigated in the rat (Parkes and Vale, 1993) in which it was found that incubation of isolated pituitaries with NEI but not MCH causes a significant reduction in AVP release whereas both MCH and NEI can independently stimulate oxytocin release. In sheep an icv infusion of MCH is followed by a small but significant increase in plasma osmolarity (Parkes, 1996) that might be expected to trigger an anti-diuretic response. In fact, serum AVP levels were unaffected although plasma aldosterone decreased and diuresis increased. The author suggests that MCH/NEI and AVP may be colocalised within magnocellular neurons and that modulatory effects may therefore be exerted locally in an autocrine or paracrine fashion.

modulatory effects may therefore be exerted locally in an autocrine or paracrine fashion. Interestingly, MCH has recently been shown to inhibit the epithelial short circuit current across frog skin stimulated by an AVP analog but has no effects when applied alone (Smriga *et al.*, 1994). It is suggested that the peptide effects this inhibition by either reorganising the membrane structure or by modifying AVP receptor structure or binding capacity. Hence there is some evidence to show that MCH may inhibit AVT peripherally in amphibians, and in these and other animals could also act centrally.

There have been several investigations into the effects of dehydration and hyperosmolarity on MCH in higher vertebrates. In rats given 2% saline in drinking water for up to six days, there is a slight and transient decrease in MCH mRNA synthesis and an increased immunoreactivity in the hypothalamus and pituitary (Fellmann *et al.*, 1993), the latter confirming earlier observations (Zamir *et al.*, 1986). In a similar study, saline administration again led to an overall ppMCH reduction in both male and female rats but did not affect all groups of MCH neurons in the same way since MCH synthesis was depressed in the medial hypothalamus but stimulated in the *zona incerta* and posterior hypothalamic MCH neurons (Presse and Nahon, 1993). Dehydration for 24 h leads to a depression of MCH mRNA in female rats and variable responses in males (Presse and Nahon, 1993) whereas water deprivation for 8 d results in a 50% increase in hypothalamic MCH mRNA content (Fellmann *et al.*, 1993). These conflicting observations may reflect the operation of diverse inputs to the MCH neuronal system, for example increased osmotic pressure, hypovolaemia, stress etc.

In summary, *Rana temporaria* has been shown to respond to osmotic challenge by increased MCH neuronal activity. This response may be due to dehydration, increased osmotic pressure and/or stress. Similar observations in mammals suggest that an osmoregulatory role for MCH may have been conserved through evolution. An association between MCH, its copeptides and neurohypophysial hormones has been established in mammals and may exist in amphibians. In anurans, the perikarya of AVT neurons are located in the magnocellular PON, a region richly innervated with MCH nerve fibres and both MCH and AVT fibres are seen in the median eminence. In addition, there is evidence to show that MCH can inhibit AVT-induced sodium transport in frog skin. It is proposed, therefore, that a principle osmoregulatory role for MCH may be in the central control of AVT synthesis or release.

Throughout the previous experiments MCH cell bodies were only seen in the posterior hypothalamus, in the infundibular region in either *Rana temporaria* or *Xenopus laevis*. This distribution accords with previous published findings in these species (Baker, 1988) and also that in Couch's spadefoot toad, *Scaphiopus couchii* and the cane toad, *Bufo marinus* (unpublished observations). Between them, these represent members of four amphibian families, respectively Ranidae, Pipidae, Pelobatidae and Bufonidae. However, in *Rana ridibunda* the largest group of MCH neurons is reported to be in the mid and caudal regions of the pre-optic area whilst only a few neurons are found in the NID region (Andersen *et al.*, 1986). In this species immunoreactive fibres are also present in the internal zone of the median eminence and the neurohypophysis. In order to determine whether or not the MCH distribution shows a genuine species difference sexually immature, adult *Rana temporaria* were used to map immunoreactive cell bodies and fibres throughout the brain.

In sexually immature *Rana* the irMCH cell bodies were located exclusively in the infundibular nuclei with fibres coursing to the olfactory lobes, optic tecta, habenular nucleus and along the ventral floor to the median eminence. In sexually mature females, however, irMCH perikarya were seen also in the anterior PON and the NLS of the telencephalon although the fibre tracts were similarly distributed. Since the numbers of immunoreactive cells in both loci were significantly diminished in spent females it is probable that the activity of these neurons is linked with reproductive function. It has not been established if the staining in these novel locations was due to increased synthetic activity or to a buildup of peptide resulting from an inhibition of release.

The pre-optic area of the amphibian brain is a site where numerous other peptidergic neuronal cell bodies are located including  $\alpha$ -MSH (Benyamina *et al.*, 1986) CRF (Tonon *et al.*, 1985) AVT (Andersen *et al.*, 1992) and GnRH (Crim, 1985). This area in amphibians has been compared with the periventricular preoptic nucleus in rats (Andersen *et al.*, 1986) in which ppMCH derived peptides are detected in this area of the rat brain for only a brief period in the female rat (Knollema *et al.*, 1992). During the second and third weeks of lactation only, nursing dams consistently express ppMCH immunoreactivity in the medial preoptic nucleus, periventricular preoptic nucleus and paraventricular nucleus of the hypothalamus. The authors of this work determined that MCH is not likely to be associated with the magnocellular oxytocinergic system since they could find no evidence of colocalisation nor even of congruence between the two cell groups and they conclude that MCH has an as yet unspecified role in lactation or maternal behaviour.



There may be a functional comparison between the transient appearance of PON MCH cells in the egg-carrying *Rana* and ppMCH gene expression in the preoptic area of the lactating rat. In frogs the PON group of MCH cells is no longer stainable after the eggs are shed and could not be attributed to maternal behaviour in this species. In addition, it is not known if the activity of PON MCH neurons is transient in male *Rana ridibunda* or if it seen at all in male *Rana temporaria*. Interestingly, AVT is associated with the time in amphibian reproduction when a gravid female is ready to breed, since systemic injections of AVT suppress the release call of female anurans thus facilitating successful mating (Diakow, 1978). It is thought that AVT-induced water uptake causes the female to swell and only when she reaches a particular size will the release call be suppressed and males accepted. An involvement of MCH in this mechanism would be worthy of investigation.

In both gravid and spent female frogs, a new group of irMCH cells was identified in the NLS which, like the PON irMCH neurons, waned in activity after the shedding of eggs. The NLS group covered an area of about 500  $\mu\text{m}$  along the anterior to posterior axis, flanking the first/second ventricles. The fibres from these cells may well contribute to the thick hypothalamo-hypophyseal nerve tract that courses along the ventral floor from the telencephalon to the pituitary. Since some fibres also extended medially and dorsally from this area these cells may be responsible for the previously observed irMCH tract to the habenular nucleus. The transient appearance of NLS MCH neurons during a time of sexual maturity suggests a function linked to reproduction.

One of the principle reproductive hormones in amphibians is gonadotropin-releasing hormone (GnRH) which in these animals may exist in two or three forms including salmonid

and mammalian GnRH (Sherwood *et al.*, 1986). The distribution of GnRH can differ slightly between species but generally the cell bodies are found in the telencephalon, in the septal nucleus and diagonal band of Broca, and in the preoptic area. From these cells, fibres course ventrocaudally to the infundibulum and median eminence (Sotowska-Brochoka and Licht, 1992). The activity and concentration of GnRH in the brain varies in relation to the reproductive cycle of both male and female amphibians, such that it is low in sexually quiescent animals and increases towards the start of the breeding season (Andersen, 1992). In rough skinned newts the plasma levels of GnRH are high at the initiation of courtship and fall rapidly after sperm transfer (Propper and Moore, 1991). In *Xenopus* during the months of March and April the PON group of irGnRH cells disappears with a concomitant drop in plasma androgens triggering the end of reproductive activity (Zöeller and Moore, 1985). Apart from stimulating the release of sex hormones GnRH also exerts behavioural effects. In male roughskin newts, central administration of GnRH induces clasping behaviour and makes female *Xenopus* more sexually receptive to males (Andersen *et al.*, 1992).

The MCH and GnRH systems share a similar distribution of cell bodies and fibres in the septal nucleus and preoptic area. It is therefore possible that both hormones are influenced by the same factors or that MCH may modulate the activity of GnRH neurons at one or both loci. Interestingly there exists a small group of ependymal cells in the amphibian telencephalon that show immunoreactivity with 17 $\beta$ -hydroxysteroid dehydrogenase, an enzyme required for the formation of testosterone and oestradiol. These gliocytes are located in the lateral and medial septal nuclei in *Rana ridibunda* (Mensah-Nyagan *et al.*, 1996) and could perhaps form connections with both MCH and GnRH neurons.

In conclusion, this chapter has initiated new avenues of enquiry concerning the role of MCH in osmoregulation and reproduction. It has been demonstrated that the MCH system is sexually and functionally dimorphic and that, like many hormones, its activity is attuned to variations in season and physiological condition. The amphibian has proved to be a good model to investigate the functions of this ubiquitous peptide and has, perhaps, provided fuel for continuing investigations on the role of MCH in higher vertebrates.

## CHAPTER 5

### THE EFFECT OF SALINITY ON HYPOTHALAMIC MCH IN THE RAINBOW TROUT

#### 5.1 Introduction

A role for MCH in the process of osmoregulation has been proposed in mammals. Rats given 2% saline in the drinking water for periods up to 6 d show an overall depression of MCHmRNA synthesis in the majority of neurons and increased MCH immunoreactivity (Presse and Nahon, 1993; Zamir *et al.*, 1986). Dehydration for 1 d has the same effect as salinity in one report (Presse and Nahon, 1993) but significantly enhances MCHmRNA after 8 d in another study (Fellmann *et al.*, 1993).

The MCH neurons also appear to be responsive to osmotic challenge in lower vertebrates. The first emergence of young frogs onto land after metamorphosis coincides with an increase in MCH synthetic activity which may reflect dehydration, since the aquatic *Xenopus* does not show this response at the same developmental stage (Chapter 3). Similarly, saline treatment in adult *Rana* enhances irMCH neuronal activity (Chapter 4). In contrast to the effects in amphibians and mammals, the MCH neurons in the teleost fish *Oreochromis mossambicus* failed to show any change in MCH mRNA levels after a 70% seawater challenge for 10 d (Gröneveld *et al.*, 1995b). Hence, as with mammals, the effect of osmotic stimuli on MCH activity in lower vertebrates is unpredictable.

## Chapter five

Teleost osmoregulation depends on the integrated activity of the epithelial surfaces of the gills, gut and renal system which allows the fish to maintain the osmolarity of their body fluids at levels significantly different from the external environment. In a hypoosmotic environment fish drink very little but gain a lot of water, which enters passively across the body surface. Ions are actively pumped into the body at the gills whereas the body surface is ionically impermeable to prevent loss. A high glomerular filtration rate (GFR), together with tubular and bladder reabsorption of ions, results in the production of copious quantities of a highly dilute urine. In a saline environment the osmotic loss of water is compensated by drinking, absorption in the gut, and a reduction in the glomerular filtration rate. Excess ions, which enter passively and in swallowed sea water, are lost by active extrusion at the gills. Of all the epithelial surfaces the gills represent the most extreme form of functional perturbation since a change of environment can cause a complete reversal in the direction of NaCl transport (reviewed in Foskett *et al.*, 1983). In salt water, teleost branchial chloride cells, powered by  $\text{Na}^+/\text{K}^+$  ATPase, actively pump out chloride ions followed by passive movement of sodium via paracellular channels (Karnaky, 1986).

In the rainbow trout, MCH is produced in two paired nuclei, the *nucleus lateralis tuberis* (NLT) from which magnocellular neurons project mainly to the pituitary neural lobe, with fewer projections into the brain, and a second, smaller group of parvocellular neurons located above the lateral ventricular recess (LVR). The axons from this latter group project dorsally into the thalamus and make no apparent contribution to the pituitary MCH content (Baker *et al.*, 1995). In trout, both groups of neurons respond similarly to changes in environmental colour but in both trout and tilapia the two MCH populations show divergent responses to some forms of stress. Thus it has been found that only the NLT neurons show

a significant increase in ppMCHmRNA following exposure to shallow or acidified water whereas the LVR-MCH neurons remain unaffected (Gröneveld *et al.*, 1995b; Suzuki *et al.*, 1996).

The current work quantifies the progressive changes in trout MCH mRNA, both in NLT and LVR-MCH neurons, in response to increasing concentrations of seawater and correlates these with plasma osmotic pressure and cortisol concentrations. The response of MCH neurons to saline challenge and the relationship between MCH and other osmoregulatory hormones is discussed.

## **5.2 Materials and methods**

### **5.2.1 Experiment 1.**

Forty mature trout (average body weight 200 g) were obtained from Alderley Trout Farm, put into three 250 litre tanks and left for 10 d to acclimatise. The aquarium conditions and feeding regime were as outlined in Chapter 2 (section 2.1.1).

Salinity in two tanks was adjusted by siphoning off fresh water and replacing it, at 40 litres per hour, with appropriate quantities of previously prepared, full strength seawater (16.13 kg salt in 400 litre water. Instant Ocean, Aquarium Systems, Sarrebourg, France) to give 50‰ salinity (measured at 502 mOsm). Water quality was maintained by circulating the tank contents through a biological filtration system. After 24 h six fish were removed, and killed, and the rest were left to equilibrate for 6 d, after which the salinity was adjusted to 80‰ (796 mOsm). Again, six fish were killed after 24 h exposure and the rest left for 6 d. Finally, enough sea salt was added to bring the seawater to full strength (1000 mOsm). Six

fish were killed after 24 h and a further six after 6 d. Six control fish were killed on the first and last days of the experiment. Water exchanges were achieved quietly, and without removing the tank covers, in order to minimise disturbance. Fish were killed, as near as possible, at the same time of the day on each occasion in order to reduce the effects of diurnal expression of MCH.

Following anaesthesia in 0.06% (v/v) phenoxyethanol blood samples were taken from the caudal vein and the plasma was retained for cortisol radioimmunoassay and determination of plasma osmolarity. The brains were then exposed and perfused with 4% paraformaldehyde fixative before removal. After overnight postfixation, dehydration and wax embedding, sections were cut at 10 µm and mounted on chrome-alum subbed slides.

The *in-situ* hybridization procedure is described in Chapter two (sections 2.7.3 to 2.7.5) and was followed here. The sections were incubated at 37°C for 18 h with a <sup>35</sup>S-labelled oligonucleotide probe, complementary to the 3' untranslated region of MCH1 or MCH2 mRNA (see Figure 14).

**MCH1: 5' GAAAGCGAAAACATATACATAAGTAC**

**MCH2: 5' AGATGATACGACGTAGAACAGGACGGAG**

Fig 14. MCH Oligoprobe sequences

Sections were washed in 1xSSC at 60°C and exposed to autoradiographic film for 10 h (NLT) and 7 d (LVR-MCH). Total binding was assessed and the data were analysed using

the Mann-Whitney non-parametric test. Plasma cortisol levels were measured by standard cortisol RIA (Chapter two, section 2.5) and plasma osmolarity by freezing point depression. Data for cortisol and osmotic pressure were analysed using the two-tailed Student's t-test.

### 5.2.2 Experiment 2

This experiment was conducted as before, but fish were not sampled at 50% salinity. Instead, six samples were taken after 24 h and 6 d in both 80% and full strength seawater. The *in-situ* hybridization was performed as before, using a probe against trout MCH2 and, in addition, with a similarly labelled probe complementary to MCH1 mRNA. Sections were washed in 1xSSC at 60°C and exposed to autoradiography film for 10 h (NLT) and 7 d (LVR-MCH). The slides hybridized with MCH1 probe were exposed for six weeks to increase the likelihood of observing any signal.

## 5.3 Results

The mean ( $\pm$  SEM) plasma cortisol concentrations and osmotic pressures are shown in Figure 15. The data shown are from two experiments. In control fish there were no significant changes in plasma cortisol or osmotic pressure of fish sampled on day 1 or day 21 and the data are therefore combined in the graph. There were no correlations between fish weight, plasma osmotic pressure or cortisol levels. The effect of salinity on osmotic pressure and cortisol was similar in both experiments. Plasma cortisol in FW was less than  $1 \text{ ngml}^{-1}$  and was not significantly raised after 24 h in 50% SW. After this point, levels increased significantly in each SW concentration and by the last day of the experiment, in 100%SW, had risen to between 27 and  $30 \text{ ngml}^{-1}$ .



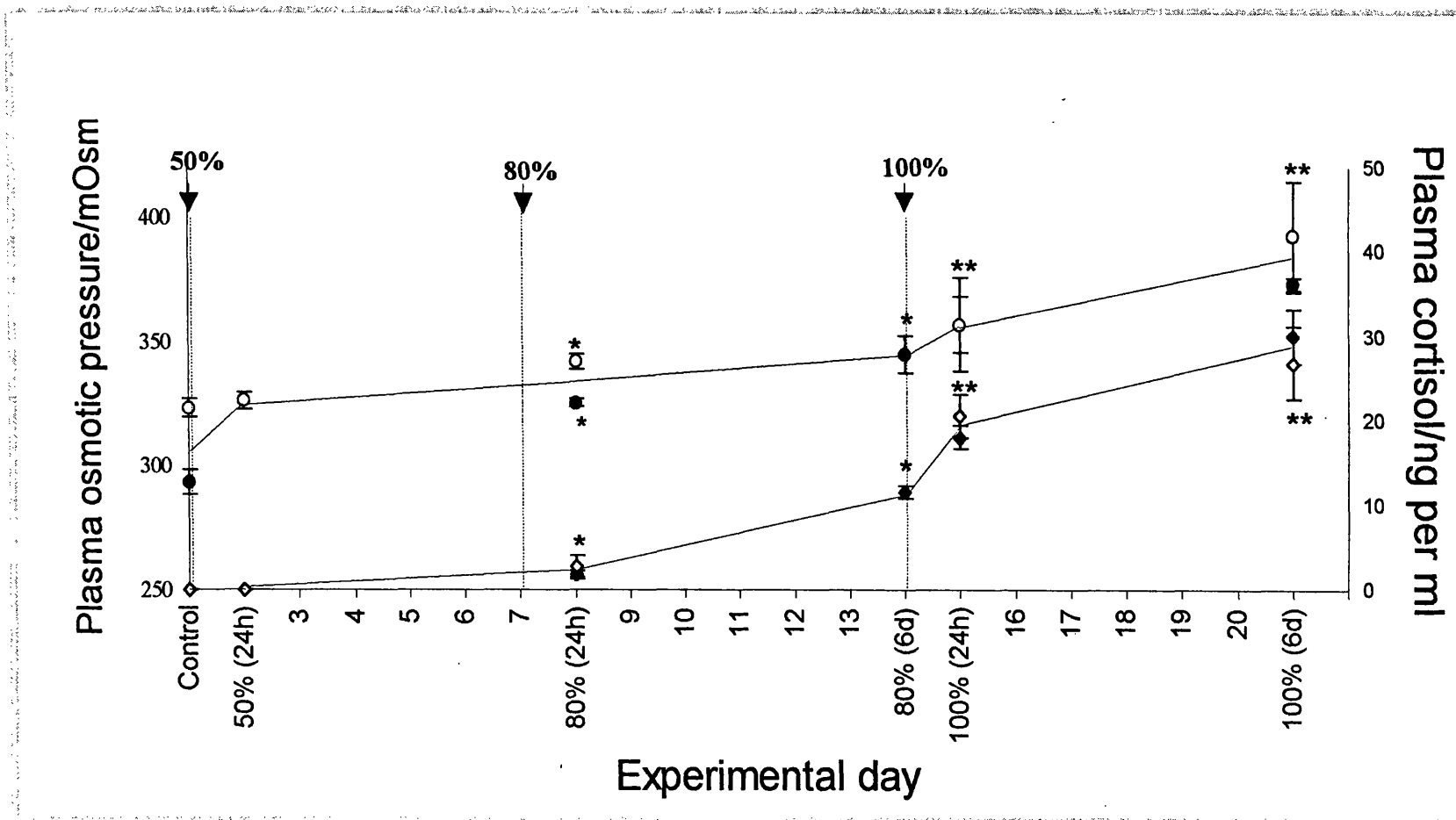


Fig 15. Changes in mean plasma osmotic pressure and cortisol concentrations in trout following seawater challenge. The results are from two experiments. Osmotic pressures are shown by  $\circ$  (experiment 1) and  $\bullet$  (experiment 2) and plasma cortisol concentrations by  $\diamond$  (experiment 1) and  $\blacklozenge$  (experiment 2). Vertical broken lines indicate the points at which each saline treatment was commenced. \*  $P < 0.01$  and \*\*  $P < 0.001$  compared with controls.

Plasma osmotic pressures, similarly, showed no significant rise from basal levels (308 mOsmkg<sup>-1</sup>) until 24 h after exposure to 80% SW (343 mOsmkg<sup>-1</sup>). After this, a steady increase was observed which, after 6 d in 100% SW, had reached a mean value of 382 mOsmkg<sup>-1</sup>, approximately 125% of controls. The sharpest increase in both cortisol and osmotic pressure occurred after 24 h exposure to full strength seawater.

Figures 16 and 17 show changes in the mean total probe binding by MCH mRNA in the NLT and LVR regions under different salinities. Since probe binding is calculated in arbitrary units these values can be highly variable between experiments. Thus, the total binding for each experiment is expressed as a percentage of control values to permit easier comparison between the two experiments, the results of which were very similar.

Figures 18a and b shows MCHmRNA binding in the control trout brain. In the NLT, there was no change in MCHmRNA until 24 h in 80% SW (Figure 18c), when it showed a significant ( $P < 0.01$ ) increase to approximately 154% (Experiment 1) and 156% (Experiment 2) of control values but the LVR-MCH neurons were unaffected. After a further five days in 80% SW the level of message in the NLT neurons had declined to control values but a subsequent 24 h exposure to full strength seawater (Figure 18d) led to a highly significant decrease in mRNA levels in both NLT (52% Experiment 1 and 72% Experiment 2;  $P < 0.001$ ) and LVR-MCH (18% Experiment 1 and 48% Experiment 2;  $P < 0.001$ ) neurons, but this response was again transitory and after a further five days in full strength seawater levels were once again similar to controls.

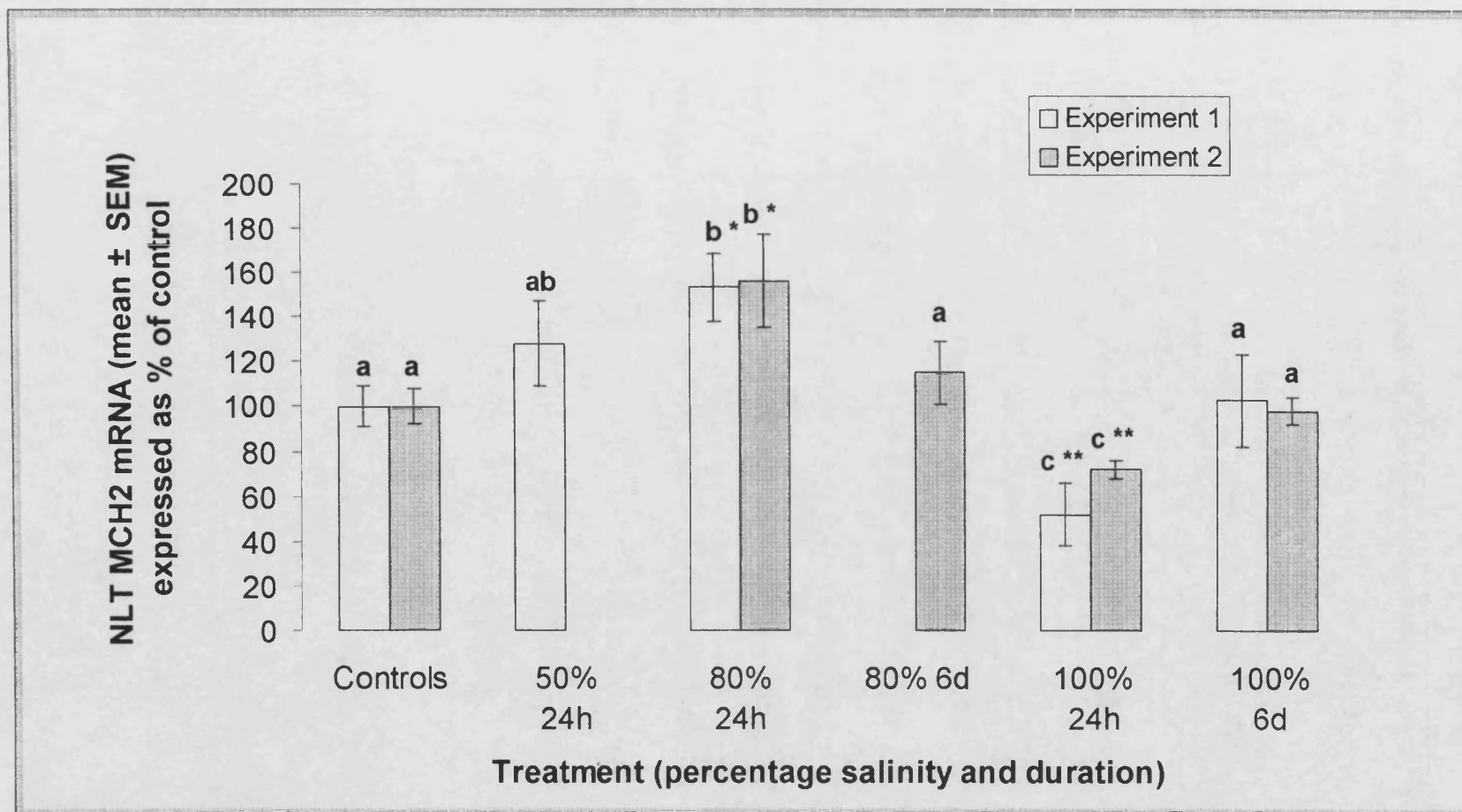


Fig 16. Mean total probe binding of MCHmRNA in the NLT region of the trout hypothalamus after seawater challenge. Results are from two experiments and probe binding is shown as a percentage of controls to allow comparison. \*  $P < 0.01$  and \*\*  $P < 0.001$ . Groups labelled with the same letters (a,b,c) are not significantly different from one another.

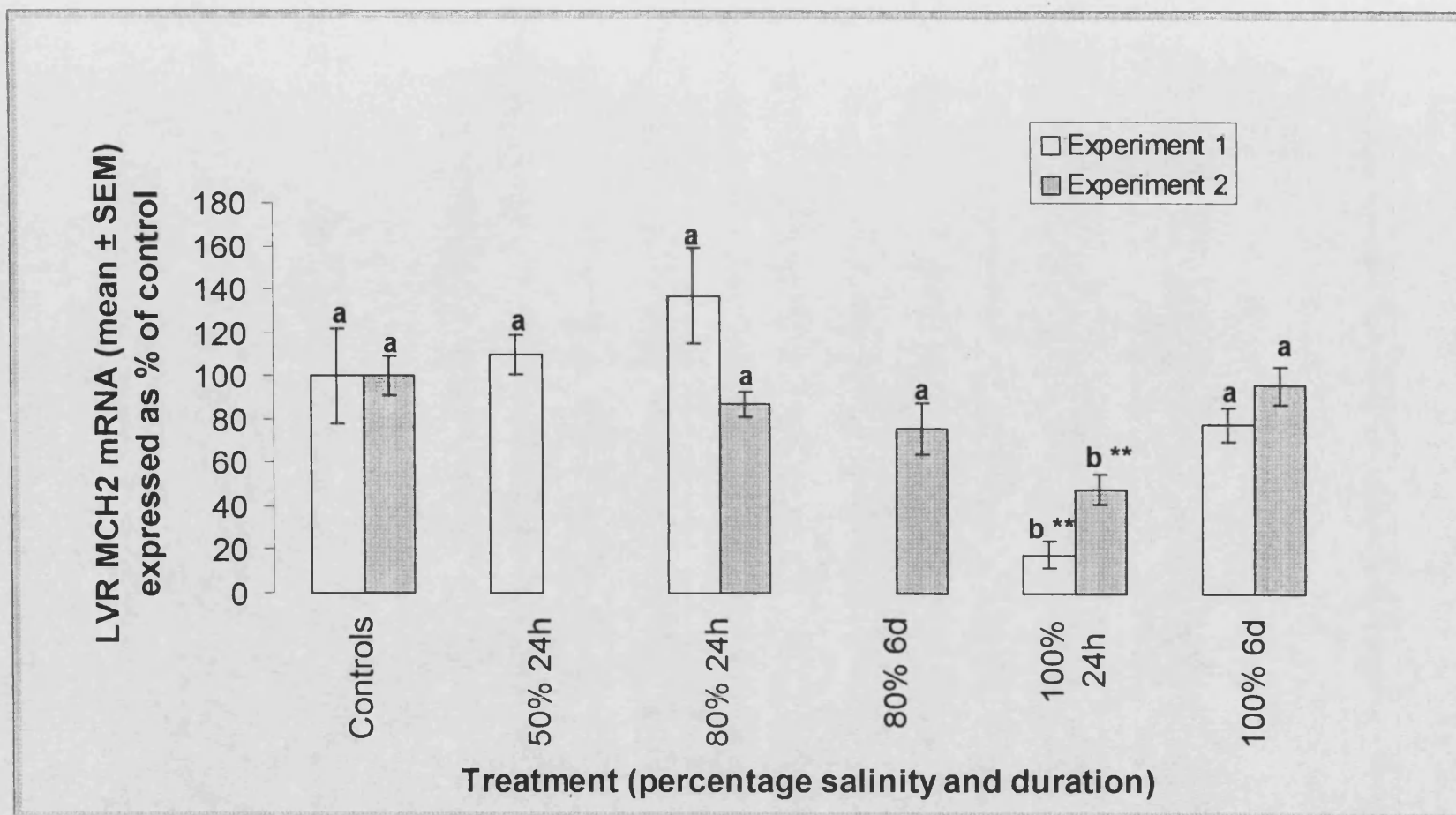


Fig 17. Mean probe binding by MCHmRNA in the LVR region of trout hypothalamus after seawater challenge. Results from two experiments are shown and the total binding is expressed as a percentage of control values to allow comparison. \*\*  $P < 0.001$ . Groups that are labelled with the same letter (a,b) are not significantly different from one another.

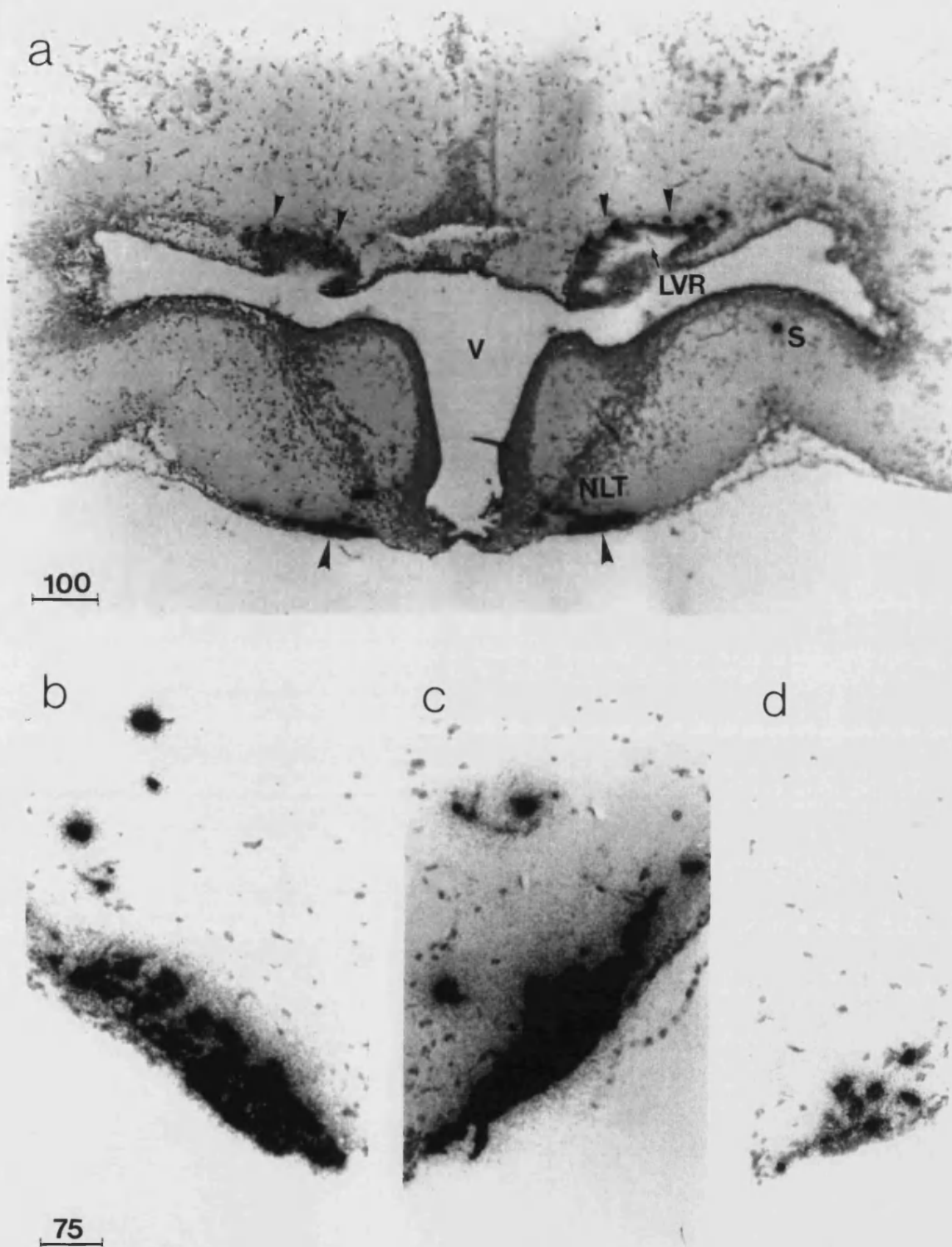


Fig 18. Transverse sections through rainbow trout brain following seawater (SW) challenge. Sections were hybridized with  $^{35}\text{S}$ -labelled oligonucleotide probe for MCH2mRNA and dipped in photographic emulsion before being developed. (a) Section showing NLT and LVR-MCH neurons (arrowed) in a control trout. LVR lateral ventricular recess, V ventricle, S spider cell, NLT *nucleus lateralis tuberosus*. (b) (c) (d) MCH probe binding in the same region of the NLT in, respectively, control, 80% SW (24h) and 100% SW (24 h) fish to show relative densities and areas of probe binding (all are at the same scale)

Thus, the NLT showed a bimodal (increase followed by decrease) response to increasing salinity whilst the LVR showed only the depressed response in 100% SW. There were no correlations between MCH mRNA levels and plasma osmotic pressures or cortisol concentrations between fish at either the zenith or nadir of MCH gene expression.

Tissue sections incubated with the MCH1 oligonucleotide probe consistently failed to show any signal either in the NLT or LVR-MCH neurons.

#### 5.4 Discussion

Progressive seawater challenge was accompanied by increases in both plasma osmotic pressure and cortisol concentration. Cortisol is required for SW adaptation to stimulate  $\text{Na}^+/\text{K}^+$  ATPase activity in the gills (Richman and Zaug, 1987) and to promote the differentiation of branchial chloride cells for ionic excretion (McCormick *et al.*, 1991). In successful teleost osmoregulation, steroid levels are raised only transiently when fish start to drink seawater whilst the resulting efflux of excess ions reduces plasma osmolality (Assem and Hanke, 1981). For example, in a recent study on rainbow trout (Sakamoto and Hirano, 1991) exposed directly to 80%SW, plasma cortisol concentrations reached a peak within 24 h but by day 4 they had returned to control values. In that example, the trout were large (up to 1600 g) and appeared to osmoregulate well but in the present work the fish were smaller (~ 200 g) and showed sustained high levels of plasma osmotic pressure, suggesting an inadequate osmoregulatory ability. Although factors other than ACTH, for example angiotensin II, urotensin I and II (Arnold-Reed and Balment, 1994) and growth hormone (Young, 1988) can stimulate cortisol release directly, it is likely that the raised cortisol in the present work reflected stress arising from osmoregulatory failure.

## Chapter five

The absence of a significant rise in either plasma osmotic pressure or cortisol levels in 50%SW suggests that trout can readily adapt to this challenge, and that under these conditions there is no effect on MCH gene expression. This is similar to findings in the tilapia, *Oreochromis mossambicus* which was readily able to osmoregulate in 70% SW (Gröneveld *et al.*, 1985). Since this species can adapt to double strength seawater (Assem and Hanke, 1981), it is probable that the acclimation threshold would be higher in tilapia than in rainbow trout. The minimal response in these parameters when trout were subjected to 50% SW also suggests that the experimental protocol was not in itself stressful to the fish. This was confirmed by the fact that plasma cortisol concentrations were not significantly different between control fish sampled on the first and last days in both experiments.

As trout were subjected to progressive increases in salinity, the MCH mRNA in the NLT neurons responded in a biphasic way : an initial increase in abundance, when the cortisol and osmotic pressures were still relatively low, within the range seen during successful osmoregulation (Avella *et al.*, 1993), and a depression in abundance when plasma cortisol and osmotic pressures were markedly raised. The LVR-MCH neurons only showed a depression in 100% SW which, as in the NLT, was only a transitory response. It is not possible to know from these studies, which of the stimuli - raised plasma osmotic pressure or stress - was responsible for either phase of the MCH response.

Osmotic challenges appear to influence MCH gene expression also in rats and amphibians. In newly metamorphosed *Rana temporaria*, emergence onto land with the consequent dehydration challenge stimulates MCH cell activity as judged by cytological evidence

(Chapter 3). Similar responses were observed in adult frogs after exposure to 35% saline (Chapter 4). In rats, prolonged dehydration similarly causes an increase in the abundance of MCH mRNA (Fellmann *et al.*, 1993) but 2% saline given as a drinking solution depresses MCH gene expression (Fellmann *et al.*, 1993; Nahon *et al.*, 1993; Presse and Nahon, 1993) and peptide release (Zamir *et al.*, 1986; Fellmann *et al.*, 1993). Recently, however, rats given a mild osmotic challenge (0.9% NaCl to drink) showed enhanced rather than depressed levels of MCH mRNA (D. Fellmann, personal communication). Also, cultured hypothalamic MCH neurons responded to a hyperosmotic sucrose medium by increased MCH secretion. These results were interpreted as evidence that a rise in osmotic pressure enhances MCH gene expression whilst the decline in MCH mRNA that occurred in response to 2% saline was stress related. It has already been demonstrated that another stress, from electric footshocks, causes a 60% depression of total MCH mRNA which subsequently returns to control values within a week, despite continued stress (Presse *et al.*, 1992), probably in response to corticosteroid feedback.

If these conclusions from tetrapods are applicable to fish, then a possible interpretation of the present data is that the initial rise in MCH mRNA in the NLT neurons is a response to the osmotic stimuli to which the LVR-MCH neurons are insensitive, whilst the subsequent reduction in MCH gene expression in both sets of neurons might be a stress response. A difficulty with this interpretation, however, is that several forms of stress are known to enhance MCH mRNA in the NLT neurons of both trout and tilapia. Thus, brief but repeated periods of low water stress in adult trout (Suzuki *et al.*, 1996), or the stress of acidified water in the case of tilapia (Gröneveld *et al.*, 1995b) both enhance MCH mRNA in the NLT-MCH neurons but leave the LVR-MCH neurons unaffected. Another form of



stress, resulting from repeated, daily immersion for 1 min in ice cold water, enhances MCH mRNA in both sets of neurons in trout fry (Suzuki *et al.*, 1996). Similarly the stress of a daily ip injection of large (1 ml) volumes of isotonic saline will stimulate MCH release from the trout NLT neurons, enhancing MCH plasma concentrations, a response which is prevented by dexamethasone injection (Green *et al.*, 1991). The only prior observation that stress can depress MCH neuronal activity comes from experiments in which trout were subjected daily to both isotonic saline injection and low water stresses in response to which *in vitro* MCH synthesis was significantly lower than in fish given only saline injections (Baker and Bird, 1992). In this case, therefore, the response to stress apparently depended on its nature, intensity or frequency: injections alone caused an increase in MCH biosynthesis whereas the dual treatment, injection and low water stress, were depressive.

A possible interpretation of the current experiments, therefore, is that both the initial increase in NLT neuronal activity and the subsequent depression of MCH mRNA in both groups of neurons were due to persistent and increasing levels of stress (activation of the HPI axis) arising from an inability to maintain appropriate plasma osmotic levels rather than to osmotic stimulation *per se* of the MCH neurons. It remains possible that some aspect of the response may be attributable to negative feedback of cortisol, either on the MCH neurons themselves or onto the CRF component of the HPI axis, as proposed for rats (Parkes and Vale, 1992; Presse *et al.*, 1992).

The way in which products of the ppMCH gene might contribute to achieving osmoregulatory control or an ability to overcome stress is not yet known. In the rat, in which a limited number of MCH fibres project to the posterior pituitary, *in vitro* studies

suggest that both MCH peptide and a post-translational peptide product of ppMCH, termed neuropeptide-E-I (NEI), enhance oxytocin secretion by a paracrine effect on the oxytocin nerve terminals (Parkes and Vale, 1993) while vasopressin release is depressed by NEI but not by MCH. A physiological involvement of the MCH system in neurohypophysial hormone release in rats seems possible. In trout, abundant irMCH neurons from the NLT project to the posterior pituitary and hence might modulate AVT release. No significant differences are apparent between the plasma AVT concentrations of fully adapted FW and SW trout (Warne *et al.*, 1994; Pierson *et al.*, 1995) although transfer of fish from fresh water to 33% sea water results in a rapid reduction in plasma AVT concentration whereas transfer from 33% seawater to full strength SW is accompanied by a rise in plasma AVT (Pierson *et al.*, 1995). Hence the response of AVT secretion to osmotic challenge is biphasic, like NLT ppMCH synthesis, and the two events could possibly be related. In trout the gill epithelium is thought to be a direct target organ of neurohypophysial hormones (Guibbolini *et al.*, 1990) and thus derivatives of ppMCH, either MCH itself or the gene related peptide, MCHgrp (Bird *et al.*, 1990) may affect osmotic exchanges across the gills by a modulatory effect on AVT release, whether the proximal stimulus for ppMCH gene expression is osmotic change or stress. Based on a recent report that MCH can antagonise the stimulatory effects of a vasopressin analogue on sodium transport in frog skin (Smriga *et al.*, 1994) it is even possible that hormonal MCH, released from the NLT axons in the posterior pituitary, might affect osmoregulation peripherally.

A final consideration is the potential relationship between MCH and growth hormone (GH) release. In trout, exposure to 80% SW, results in a rapid increase in GH secretion (Sakamoto and Hirano, 1991) which, it is thought, acts via the liver to produce insulin

growth factor-I, which reduces plasma sodium levels after seawater exposure (McCormick *et al.*, 1991). Growth hormone also enhances the *in vivo* response of the trout interrenal to ACTH, resulting in increasing levels of plasma cortisol (Young, 1988). Whilst a recent study has indicated that rMCH has no effect on either basal or stress-induced GH release in adult rats (Bluet-Pajot *et al.*, 1995), sMCH appears to have a positive and long-lasting effect on plasma GH content in young rats (Kawauchi *et al.*, 1993). An influence of MCH on GH release and osmoregulation may exist in fish, although there are currently no data to support this idea.

## CHAPTER 6

### AN ANALYSIS OF THE *IN-SITU* HYBRIDIZATION METHOD

#### 6.1 Introduction

In the last chapter, two *in-situ* hybridization experiments were outlined and the details of the method adopted in this laboratory have been given in the Materials and Methods section (2.7). One drawback to the methodology is that it is very time consuming, since it takes two days to wax embed the tissue and several weeks to cut and mount all the brain sections. The *in situ* experiments from Chapter five involved a total of seventy-two fish, in excess of fourteen hundred prepared slides, and each experiment took three months to complete. In similar studies with rats it has been possible to cut frozen sections of several brains in one day and to process them immediately. In addition, the cytoarchitecture of the rat brain is such that representative sections, containing MCH loci, can be selected at well defined landmarks thereby avoiding the necessity of using so many slides in the *in-situ* procedure. The purpose of this chapter is to analyse the method used in this laboratory and discuss whether there may be less time consuming ways to achieve equivalent results. The focus is mainly on the selection of appropriate sections for measurement and in the preparation of those sections.

#### 6.2 Method

After fixation, trout brains are wax embedded and sections cut at 10  $\mu\text{m}$ . The entire hypothalamic area is cut and the wax ribbon examined to determine the anterior and

posterior limits which the MCH neurons are known to be contained. The anterior limit of the LVR-MCH neurons is defined by the presence of the lateral ventricular recess. Sections collected in front of this landmark belong only to the NLT neurons but sections behind it contain both NLT and LVR-MCH neurons (Figure 19).

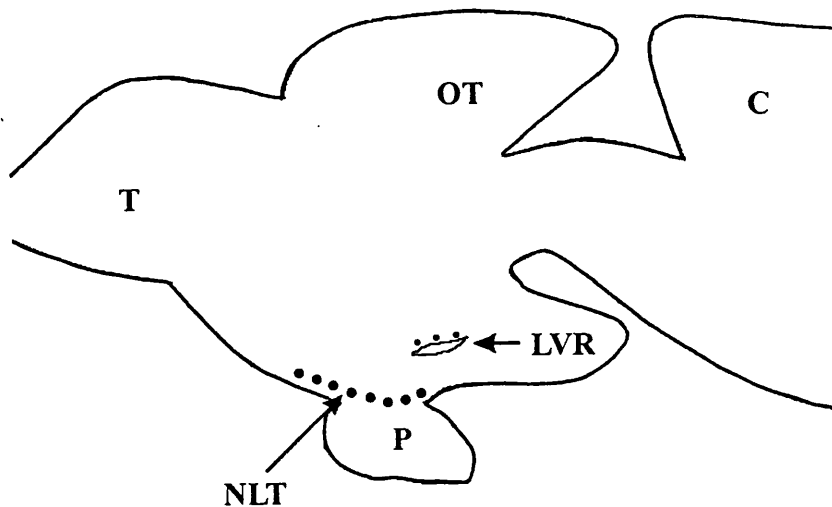


Fig 19. Schematic drawing of sagittal section through trout brain to show comparative positions of NLT and LVR-MCH neurons in the hypothalamus.

Working in the posterior to anterior direction, pairs of sections are collected and mounted serially across alternate slides, four section pairs to each slide (Figure 20). The anterior to posterior range covered by the LVR-MCH neurons is such that generally four duplicate sets are obtained. After this point the sections contain only NLT neurons which, being a far larger group of cells, are present in a greater number of sections. These, too, are collected in pairs but mounted across a set of four slides. The area covered by NLT neurons usually spans three sets of four slides. For 200 g fish, the MCH neurons extend over a distance of about 1600  $\mu\text{m}$ .

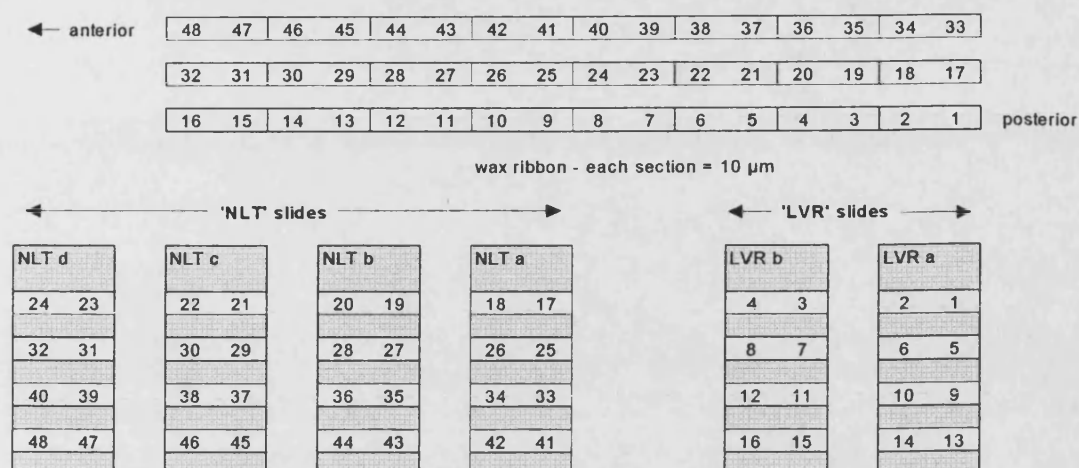
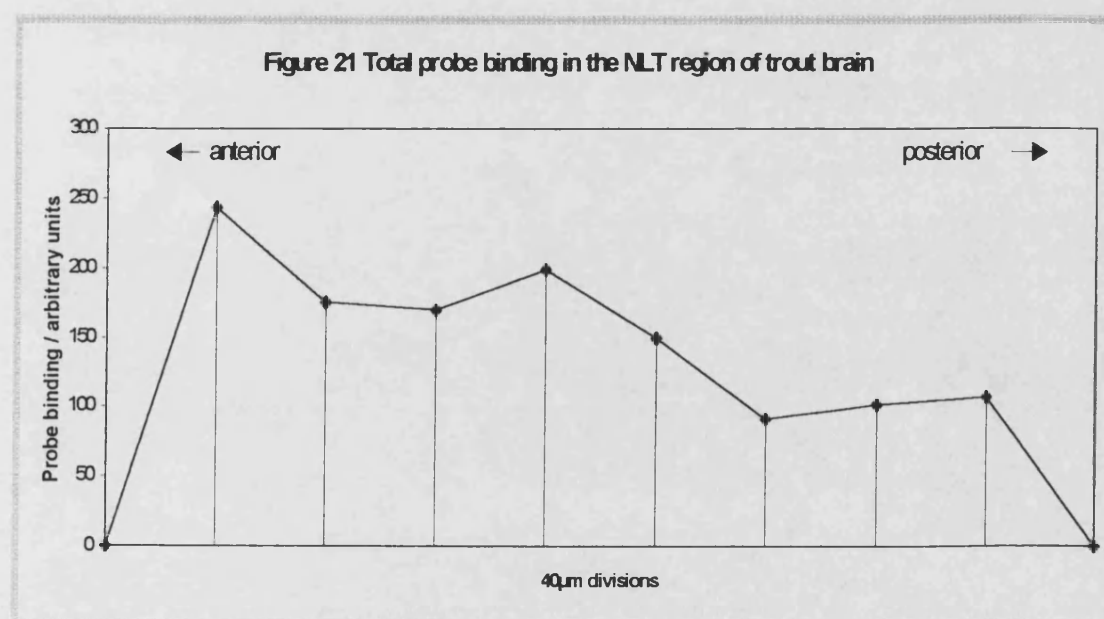


Figure 20. Mounting trout brain sections. Sections in the LVR region are mounted in pairs across two slides (usually four sets ie. 4 x 16 sections) whilst sections in the NLT region are mounted in pairs across four slides (usually two or three sets ie. 2 x 32 or 3 x 32 sections).

Following hybridization, probe binding is calculated as the product of the mean area and mean density of the signals from each pair of tissue sections. The total binding for one fish is then the sum of binding on all the pairs of sections. An example of data for one fish is shown in Figure 21.



The total binding derived from the sum of data from all section pairs is equivalent to calculating the area under the curve, as shown in the figure. However, this requires that the X-axis 'units' are equidistant. Since some NLT MCH neurons span both 'NLT' and 'LVR' slides and, due to the mounting method outlined above, the distance between successive pairs of sections on 'NLT' slides is twice that of those on 'LVR' slides. To surmount this problem the signal from NLT MCH neurons is only measured on alternate pairs of sections on 'LVR' slides. When mounted correctly the LVR-MCH signal never appears on 'NLT' slides.

In order to discuss the results (section 6.3.5) an analysis of all the signal areas, densities and probe binding from both experiments was performed, using Student's t test.

### **6.3 Results and discussion**

Figure 22 shows a sample worksheet from a group of control fish with calculations of probe binding in the NLT region, for individual fish and for the group. These data show the considerable variation between fish, as might be expected, between pairs of sections and even (not shown) between adjacent sections, due to the erratic distribution of the magnocellular neurons, which are about 8-10  $\mu\text{m}$  in diameter. If the variance in probe binding between fish is very low, then this probably indicates that the slides have been over-exposed on the autoradiographic film. Trial and error has shown that with MCH2 probe, a concentration of approximately  $1 \times 10^7$  CPM  $\text{ml}^{-1}$  and an exposure time of 10 h (NLT sections) and 7 d (LVR sections) is optimal.

ctrl1			ctrl2			ctrl3			ctrl4			ctrl5			ctrl6		
mean	mean		mean	mean		mean	mean		mean	mean		mean	mean		mean	mean	
density	area	bind	density	area	bind	density	area	bind	density	area	bind	density	area	bind	density	area	bind
0.000	0	0	0.000	0	0	0.000	0	0	0.000	0	0	0.000	0	0	0.000	0	0
2.106	9	19	2.102	11	22	2.356	53	124	2.485	63	155	2.343	38	88	3.158	77	243
2.039	11	21	2.486	22	53	2.751	48	131	2.897	33	96	2.461	26	64	3.125	56	175
2.137	20	43	2.710	24	65	2.374	33	77	3.026	17	51	2.547	23	59	2.961	57	169
2.438	25	60	2.326	25	58	2.232	32	70	2.677	8	21	2.167	9	20	3.013	66	199
2.211	18	39	2.357	29	67	2.265	30	67	2.360	33	77	2.806	46	129	2.685	56	150
2.267	16	36	2.251	34	75	2.065	28	57	2.622	43	111	2.977	46	137	2.428	38	91
2.182	12	26	2.641	29	75	2.235	33	74	2.793	39	108	2.798	43	120	2.525	40	101
2.148	19	40	2.300	37	85	2.223	30	66	2.787	40	111	2.553	27	68	2.743	39	107
2.162	27	57	2.266	34	76	1.914	3	5	2.307	40	91	2.603	42	108	0.000	0	0
2.123	19	40	2.304	33	75	1.974	15	29	2.577	43	110	2.413	39	94			
1.954	13	25	2.135	26	54	0.000	0	0	2.243	20	45	2.251	28	63			
2.021	14	28	1.882	6	10				2.151	21	44	2.486	27	66			
0.000	0	0	0.000	0	0				0.000	0	0	0.000	0	0			
<b>TOTALS</b>	<b>435</b>			<b>717</b>			<b>703</b>			<b>1021</b>			<b>1015</b>			<b>1235</b>	
<b>GROUP BINDING (mean <math>\pm</math> SEM)</b>						<b>854</b>			<b><math>\pm</math> 121</b>								

Figure 22 Sample worksheet to show calculation of total binding for a group. For each fish, probe binding = mean area x mean density from each section pair

### Why use the *in-situ* hybridization method ?

Comparing levels of mRNA from different treatment groups can be accomplished in less time using a Northern blotting procedure. However, *in-situ* hybridization has several advantages not shared by Northern blotting, for example in reliable quantification. The *in-situ* method can also distinguish between heterogeneous cell populations, which is particularly valuable if they cannot be physically separated. An example of this is found in



a Northern blot study in rats, in which it was revealed that 2% saline in the drinking water for 6 d resulted in an overall ppMCHmRNA reduction in the hypothalamus (Nahon *et al.*, 1993). A similar investigation, using *in-situ* hybridization, showed that whilst this was true for most hypothalamic MCH neurons, some cells in the *zona incerta* and around the capsule and fornix responded by increasing the MCH message (Presse and Nahon, 1993). In trout, the two groups of MCH neurons are technically difficult to separate, although it is claimed that this can be done readily in other fish species, for example in the tilapia (Gröneveld *et al.*, 1985b). Additionally, in trout, the basal hypothalamic region also contains large immunoreactive, spider-like irMCH cells (Chapter 5, Figure 18a) whose activity has not been considered previously or included in the present studies.

#### **Why use wax-embedded sections ?**

The process of wax-embedding and sectioning is time consuming whereas frozen sections can be prepared very quickly. There are three main reasons to justify using wax sections in the current experiments. Firstly, the two *in-situ* studies described in the previous chapter involved a total of seventy-two trout, the brain sections of which were stored at room temperature until required. Frozen sections must be stored between -40°C and -70°C, taking up valuable space in the freezer and should the sections thaw, for example during a power cut, then much time and material is lost. Secondly, trout brains, even when fixed, are more friable and liable to disintegration and distortion than rat brains during processing. The obvious advantage of wax embedding is to provide a solid matrix in which the tissue is supported and protected. Lastly, in order to select and mount the appropriate sections containing MCH signal, it is desirable that the brain is cut in the correct orientation,

particularly in the perpendicular plane and about the midline. The fine adjustments required to achieve such precise orientation are not as easy to achieve with frozen sections.

### **Why cut and mount all the sections ?**

One reason that an entire brain is cut and mounted is that replicate sets can be generated, enabling parallel investigations with different probes on the same fish, for example *in-situ* hybridization with probes against MCH1, MCH2 and POMCmRNAs, or for immunocytochemistry. For a pilot experiment it would be possible to dispense with replicates and mount only a single set but this would be a waste of material and, if for some reason the *in-situ* procedure did not work, would not allow the process to be repeated.

### **Data analysis**

Figures 23, 24 and 25 show data from the first *in-situ* experiment on the effect of salinity challenge in trout (Chapter 5). Each set of graphs reproduces data from three fish, in the control, 80% SW (24 h) or 100% SW (24 h) groups and compare either signal density with signal area or signal area with probe binding (density x area), which is the final value used to assess mRNA abundance. From the graphs of density with area, in all experimental groups, the density does not appear to vary markedly either along the anterior to posterior axis in individual fish, between fish, or between treatments. However, a statistical analysis of all the data from both *in-situ* experiments, using the Student's t-test, shows that whilst there is no significant difference between signal densities of control and 80% SW fish, those of 100% SW fish are significantly lower than those of controls ( $P < 0.001$ ). These findings are counter intuitive, since one might expect, looking at the sections (Chapter 5, Figures 18b,c) that the signal density in 80% SW would be higher than in controls. However, the

density reading is obtained as a measure of optical density, and the threshold for measurement is set in such a way that the small areas between cells, which nevertheless contain many granules, may not be distinguishable and hence the data for both the control and 80% SW sections are not statistically different. That the density in 100% SW is less than controls is apparent from looking at the sections (Chapter 5, Figure 18d) which show that there are areas around the cells that are devoid of any signal and which are therefore distinguishable by the scanning system, and that even within the cells, there are areas of low granule density.

The graphs of area and probe binding illustrate the unpredictable distribution of the magnocellular neurons, referred to previously, and why specific regions cannot be randomly selected and used to extrapolate total binding for the whole brain. Unlike the signal densities, the signal areas do vary, both along the anterior to posterior axis of individual fish and, significantly, between treatments. Since the signal densities within an experimental group are more or less uniform, it is the changes in area which best reflect the fluctuations in MCHmRNA abundance. An analysis of data from all the fish in both experiments shows that the correlation coefficient ( $r^2$ ) between signal area and probe binding is not less than 0.91 and that this relationship exists in data from both NLT and LVR-MCH sections. The reason for the change in signal areas between treatments relates to the nature of the decay energy of  $^{35}\text{S}$ . With higher probe binding, there are more high energy  $\beta$ -rays, which travel further from the signal locus and hence extend the detectable signal beyond the cell, increasing the area.

Taken together, it appears that increased mRNA synthesis (in 80% SW) is reflected in an increase of signal area but not density, suggesting that the number of irMCH cells is unchanged but the activity of those cells is stimulated. A reduction of area and density in 100% SW, together with the observation that the signal is contained in fewer sections along the anterior to posterior axis, implies that irMCH cells on the periphery of the group were either inactive or that their signal was too weak to be detected on autoradiographic film in the 10 h exposure time.

In summary, it must be acknowledged that the *in-situ* method employed, in these and other experiments at this laboratory, is time consuming, but it has provided results that can be reliably reproduced, allowing a hypothesis to be made concerning the response of MCH neurons to saline challenge. Since reproducibility of results is of paramount importance, there seems to be no obvious advantage in changing a system that has been shown to be effective.

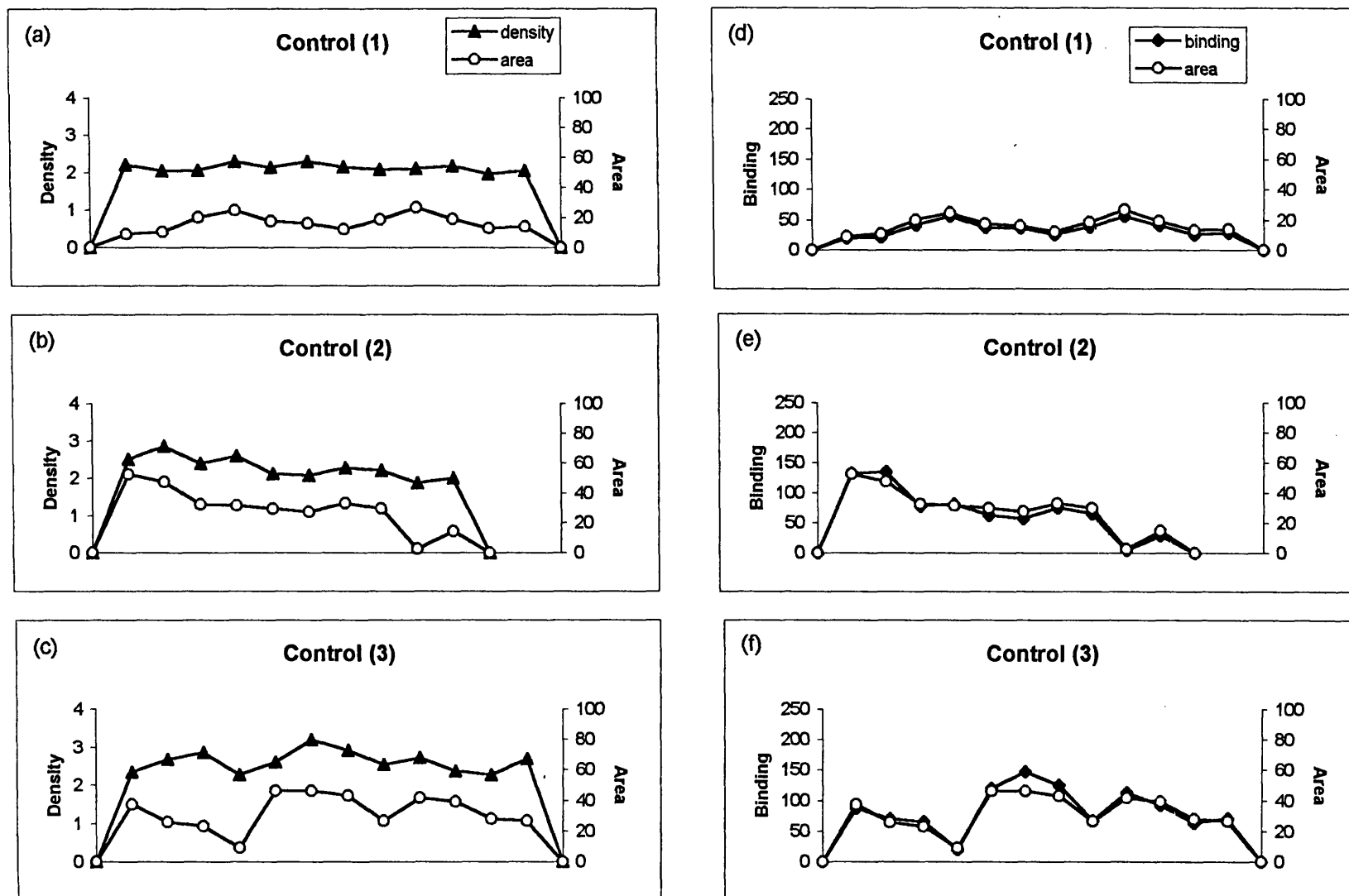


Fig 23 Graphs (a)(b)(c) show signal density and signal area from 3 control trout. Graphs (d)(e)(f) show signal area and probe binding from the same three fish. Note : all axes scales are in arbitrary units.

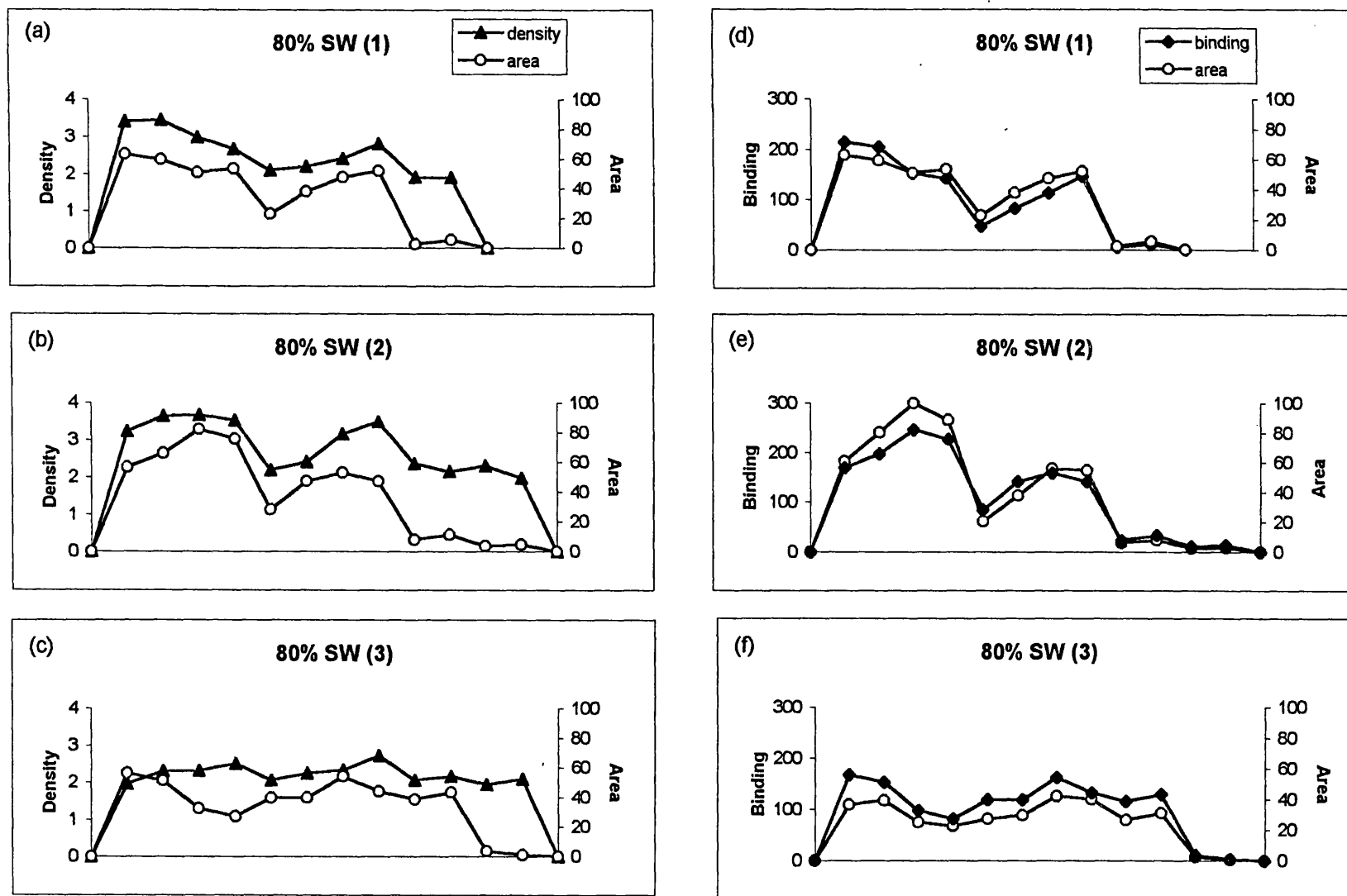
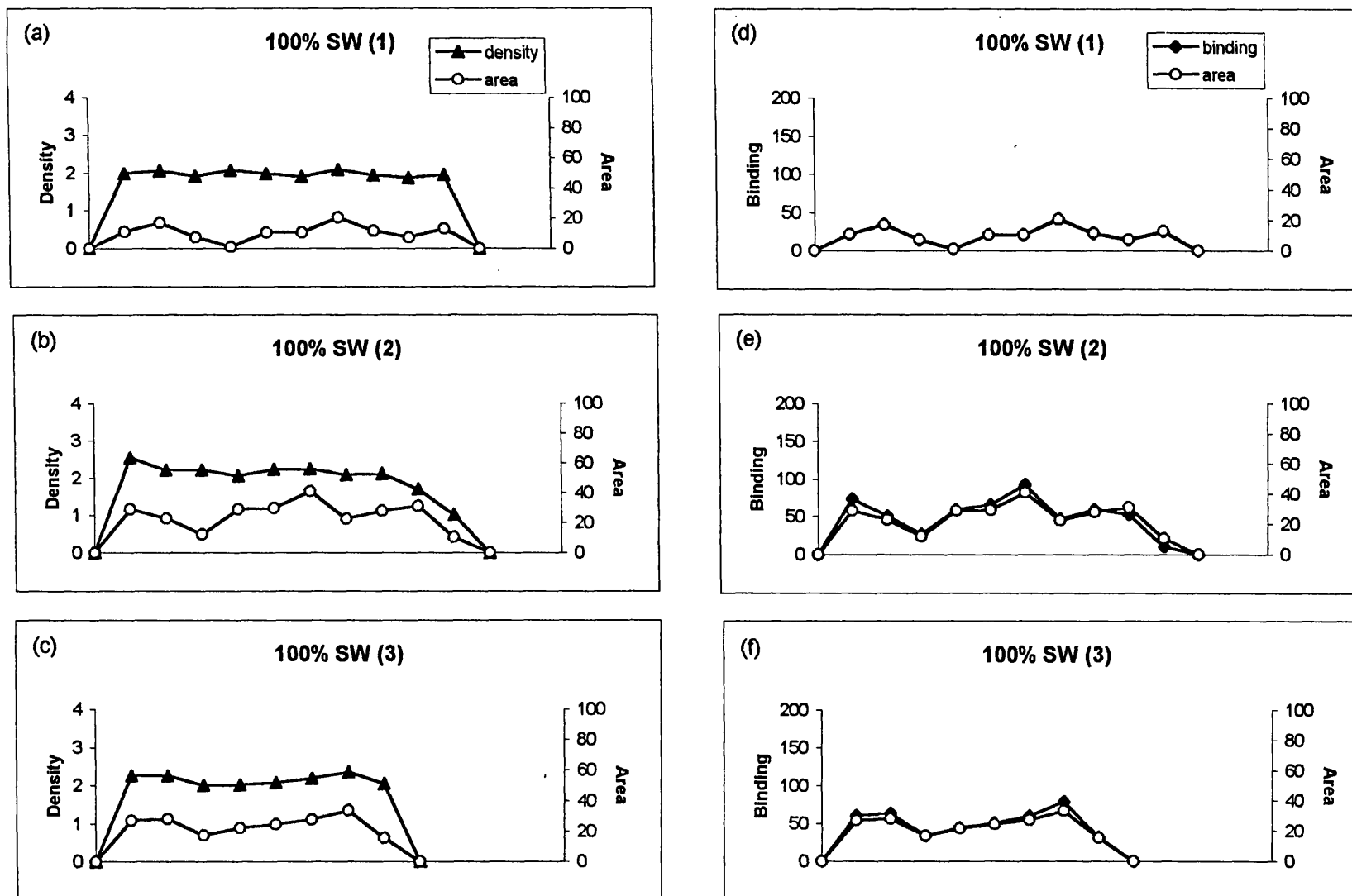


Fig 24 Graphs (a)(b)(c) show signal density and signal area from 3 trout after 80% SW (24h) challenge. Graphs (d)(e)(f) show signal area and probe binding from the same three fish. Note : all axes are in arbitrary units.



## CHAPTER 7

## MCH IN THE TROUT GUT

## 7.1 Introduction

Although in teleosts, MCH is a circulating hormone with targets at the periphery, the peptide has not been detected in the blood of mammals (Takahashi *et al.*, 1995) and is therefore believed to be predominantly a central neurotransmitter or neuromodulator, exerting its effects by controlling the synthesis or release of other hormones. In 1993 however, MCH gene transcripts, of various lengths, were detected in peripheral organs of the mouse, using a sensitive PCR technique (Breton *et al.*, 1993a). The mouse MCHmRNA was expressed in the heart, spleen, intestine and testis but in significantly lower concentrations than are found in the hypothalamus, which may be one reason why early attempts to visualise MCH in the mammalian periphery were unsuccessful (Sekiya *et al.*, 1988) or why MCHmRNA could not be detected by Northern blotting in either the salmon or rat GI tracts (Ono *et al.*, 1988; Takahashi *et al.*, 1995).

However, a later study in rats successfully revealed the presence of MCH gene transcripts in the stomach, intestine and testis and, again, the RNA species were of various lengths (Hervieu and Nahon, 1995). By *in-situ* hybridization and immunocytochemistry, MCH transcripts and ppMCH derived products were localized to the Sertoli cells of the testis and further examination showed that both MCH and ppMCH were present in the nuclei of spermatogonia and primary spermatocytes in rat, mouse, and man (Hervieu *et al.*, 1996b). In the rat gastrointestinal (GI) tract, MCH transcripts and ppMCH derived peptides were



expressed in the antral portion of the stomach and duodenum (Hervieu and Nahon, 1995), more specifically in putative plasma cells in the *lamina propria*, within and at the base of the villi, and in the submucosal layer (Hervieu *et al.*, 1996a).

It has long been established that a hormone may be present in both the brain and the gut, indeed this was found to be true for substance P as early as 1931, and since then many other hormones have been similarly identified in both regions, including cholecystokinin, vasoactive intestinal polypeptide, somatostatin and gastrin (reviewed in Polak and Bloom, 1980). Hence it was of interest to determine whether, as in mammals, peripheral MCH could be detected in fish. The current chapter describes an immunocytochemical study in three regions of the trout gut, using several variations of the technique. An attempt to detect MCHmRNA, by means of *in-situ* hybridization with a <sup>35</sup>S-labelled probe against MCH2 was unsuccessful and so the details are not included.

### 7.2 Materials and methods

Adult rainbow trout were maintained as outlined in Chapter 2 (section 2.1.1). Fish were sampled at various times and quickly anaesthetised in 0.06% (v/v) phenoxyethanol in water before being killed by decapitation. The abdominal cavity was opened and the entire GI tract removed. The gut was dissected into approximately 10 cm samples, taken from the oesophageal, pyloric and colonic regions, and quickly flushed through with ice cold 0.01M PBS, to remove any debris. The tissue was then immersed in fixative, either Bouin's or 4% paraformaldehyde (4% PFA). Each 10 cm length was sutured with cotton at one end, the

sac filled with fixative and then tied off at the other end. To aid sectioning, the outer layer of muscle was removed from all tissues after fixation.

### **7.2.1 Wax-embedded sections**

Tissue sections were fixed in Bouin's or 4% PFA for 18 h, dehydrated, cleared and embedded in wax. Serial sections were cut at 10  $\mu$ m and mounted on glycerin-albumin coated slides. Sections were immunostained for MCH using the PAP procedure, described in Chapter 2 (section 2.4.1), with either anti-rat MCH (837) at x1000 and x2000 dilutions or anti-salmonid MCH (Kawauchi) at x1000 and x2000 dilutions. Slides were incubated in primary antisera for 48 h at 4°C). After incubating in goat anti-rabbit antiserum (x25) the MCH antibody-antiserum complex was visualised by soaking slides in 0.025% (w/v) 3,3'-diaminobenzidine tetrachloride (DAB) in 0.1M HCl buffer with 100  $\mu$ l 30% (v/v) stock H<sub>2</sub>O<sub>2</sub> for approximately 5 min. Sections were then thoroughly washed before being dehydrated, cleared and mounted.

### **7.2.2 Frozen sections**

Tissue sections were fixed for 2-3 h in 4% PFA and then soaked in 10% sucrose solution overnight before being snap frozen and sectioned at 10  $\mu$ m. Endogenous peroxidases were blocked by soaking the sections in 0.3% (v/v) H<sub>2</sub>O<sub>2</sub> in PBS for 30 min before incubating for 48 h at 4°C in anti-rat MCH (837), x1000 dilution. The PAP procedure was continued as for wax-embedded sections.

Some sections were cut at 10  $\mu$ m and post-fixed for 7 min in 4% PFA before being rinsed in 0.02% (v/v) Triton-X in 0.05 PBS. These sections were immunostained for MCH using

the ABC method (Chapter 2, section 2.4.2). Sections were incubated overnight at 4°C with anti-rat MCH (837), x2000 and x5000 dilutions. After visualisation with DAB, as before, sections were counterstained in haematoxylin, cleared and mounted.

The specificity of primary antisera was determined by immunosorption. Diluted antisera were incubated overnight with MCH (~10 µg rat MCH in 250 µl diluted 837 or Kawauchi antisera or ~20µg salmonid MCH in 250 µl diluted 837 or Kawauchi antisera). Alternate sections of gut from the pyloric region were incubated overnight with normal or pre-adsorbed antisera.

### 7.3 Results

In 4% PFA-fixed, frozen sections of the pyloric region, considerable numbers of small irMCH cells were seen, using rMCH antiserum, in the layer of the *lamina propria* within and at the base of the villi, in the sub-mucosa (Figure 26b). These cells, similar in appearance to mast cells, were of an irregular shape with heavily stained, granular cytoplasm and eccentrically located, unstained nuclei (Figure 26c). Surprisingly, sMCH antiserum failed to stain these cells. In frozen sections of the oesophagus and colon, large numbers of cells were immunostained throughout the tissue together with very high levels of non-specific staining which precluded positive identification of genuine MCH-producing cells in these regions.

In frozen sections of trout pylorus, no immunoreactive cells were visible in sections incubated with preadsorbed rMCH antisera.

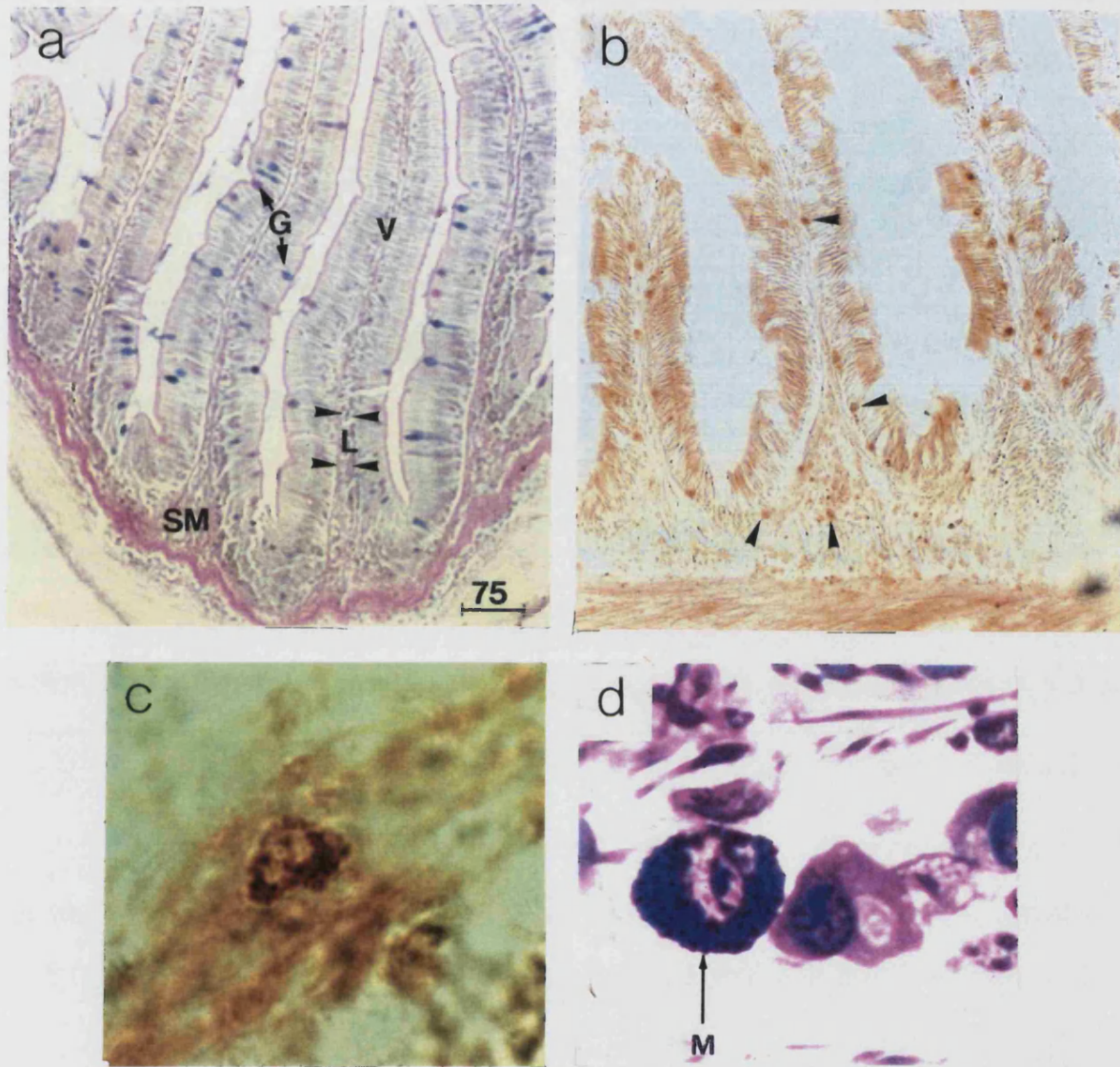


Fig 26. Immunoreactive MCH cells in the trout gastrointestinal tract. (a) Section through pyloric region of trout gut stained with periodic acid Schiff's stain and alcian blue. G goblet cells, V villus, L *lamina propria*, SM submucosa. (b) Frozen section through pyloric region of trout gut showing irMCH cells (arrowed) within and at the base of the villi in the *lamina propria* and submucosal layers. Scale bar represents  $\mu\text{m}$ . (c) Single irMCH cell under oil immersion (x1000). The nucleus is eccentrically located and the cytoplasm highly granular and irregular in outline. (d) Mammalian mast cell (x1200) stained with toluidine blue to show the similarity in morphology with 26(c). This micrograph is reproduced from Wheater's Functional Histology 3rd edition, with permission from the publisher, Churchill-Livingstone, Edinburgh, UK.

In wax-embedded sections, either in material fixed with Bouin's or 4% PFA, no immunoreactive MCH cells were seen but the background material was heavily stained, despite quenching of endogenous peroxidases and the blocking of non-specific binding with normal serum. Regions of staining were most apparent at the tips of villi, along the brush borders, suggestive of non-specific staining of mucus.

### 7.4 Discussion

In a previous study on rat, Hervieu and Nahon (1995) used tissue fixed in 4% PFA and snap frozen for immunocytochemistry and *in-situ* hybridization. They showed the presence of MCH in the antral portion of the stomach and duodenum where the majority of irMCH cells were located in the *lamina propria* and mucosal plexus and in the submucosal layer at the base of the villi. The authors suggested that the cells, of irregular shape and with eccentrically located nuclei, were possibly plasma cells. The current work shows that irMCH is present in the pyloric region of the trout gut and appears in cells, similar in appearance to mast cells, located within and at the base of the villi in the *lamina propria* and in the sub mucosa.

It has recently been shown that the localization of MCH- and atrial natriuretic factor (ANF)-containing cells are similar in the rat colon, and may co-exist in the same cells with catecholamines indicating that MCH-expressing cells are probably antigen-presenting members of the enterochromaffin cell system (Hervieu *et al.*, 1996a). In the guinea pig, ANF immunoreactivity also occurs directly beneath the *lamina muscularis* and in lymphatic nodules of the *lamina propria* and submucosa of the colon and jejunum (Vollmar *et al.*,

1988). The irANF cells are described as plasma cells on the basis of the heavily immunostained, granular cytoplasm and rounded eccentrically placed nuclei.

It has been observed that the mammalian MCH prohormone and that of ANF share a 28% identity in the C-terminus amino acid sequence and hence may be structurally related, although this similarity is not as strong in salmon (Presse and Nahon, 1991, in Nahon, 1994). Although their primary sequences are dissimilar, in binding studies on rat PC12 phaeochromocytoma cells and mouse B16-F1 melanoma cells, rat ANF (1-28) is a weak competitor of iodinated (Phe<sup>13</sup>, Tyr<sup>19</sup>)-MCH, being tenfold less potent on B16-F1 cells but half as potent on phaeochromocytoma cells, suggesting similarities in topology and perhaps, function (Drozd *et al.*, 1995). In rat, both MCH and ANF stimulate water, sodium and potassium fluxes at the level of the proximal colon and increase sodium and potassium fluxes in the duodenum (Hervieu *et al.*, 1996a). Whether these functions can be demonstrated for teleost MCH remains to be investigated.

In the rat duodenum, there is also a similarity in expression between MCH- and AVP-producing cells (Hervieu *et al.*, 1996a). Both are located in mast-like cells in the *lamina propria* and sub mucosa and, since it is found with its associated neurophysin, AVP is believed to be synthesised *in situ* rather than taken up from the blood. In the human duodenum, irAVP is again found in mononuclear cells of the *lamina propria* and sub mucosa and, it is suggested, may influence regional blood flow or modulate the actions of histamine, which stimulates chloride secretion into the lumen (Friedmann *et al.*, 1991). Although it has been shown that MCH does not modify the release of AVP from the pituitary gland *in vitro* (Parkes and Vale, 1993), the peptides may have similar targets in the

gut, and since MCH can inhibit the actions of an AVP analogue on frog skin (Smriga *et al.*, 1994) functional antagonism of AVP may be the role of MCH in the periphery.

No MCH immunoreactivity was detected in gut sections that had been either wax-embedded, fixed in Bouin's, or immunostained with an anti-salmonid antiserum, known to bind well to MCH in the brain. Although speculative, this failure to observe irMCH may be due to the following reasons: Firstly, in the current experiments, if irMCH was contained in eosinophilic granulocytes (EGCs), the fish homologue of mammalian mast cells (Dorin *et al.*, 1983) then the cytoplasmic granules, which are highly water soluble in mast cells (Burkitt *et al.*, 1993) may have been lost during the wax embedding procedure.

Secondly, it is possible that the gut form of MCH may be structurally slightly different from that found in the brain. A study on the expression of AVP production in the human GI tract (Friedmann *et al.*, 1991) revealed that the vasopressin-associated human neurophysin (VP-NHP) could be detected in the pituitary, but not in gut tissue, after Bouin's fixation and using the same antisera. The authors hypothesised that the fixative may alter the antigenicity of some proteins through cross-linking, implying that there were differences in post-translational or post-transcriptional processing between brain and gut VP-NHP. That differential processing of MCH occurs within the brain is illustrated by the fact that not all irMCH neurons cross react with  $\alpha$ -MSH antisera (Andersen *et al.*, 1987; Powell and Baker, 1987). In the gut, MCH may be structurally distinct from that found in the hypothalamus, for example as a result of the activity of proteinases other than those associated with hypothalamic MCH (Seidah *et al.*, 1993). Were this the case, then fixation by Bouin's may have altered the antigenicity of MCH to a point where the peptide was no longer detectable.

Finally, an early work showed that MCH immunoreactivity could be detected in the mammalian brain, but not in the periphery, using an anti-salmonid primary antiserum (Sekiya *et al.*, 1988). This suggests that MCH expressed in fish gut may bear a greater similarity to mammalian MCH, which is found in both the mammalian brain and at the periphery, than it does to hypothalamic MCH of the same species.

To summarise, this pilot experiment has shown that there are immunoreactive MCH cells in the trout GI tract, predominantly in the *lamina propria* and sub mucosa of the pyloric region. The cells have a similar appearance to mammalian mast cells and may therefore be the fish homologue, eosinophilic granulocytes. It appears, from the results over several experiments, that peripheral MCH in trout can best be visualised by using tissue that is briefly fixed in 4% PFA, snap frozen and immunostained using anti-rat MCH primary antisera. Much more work needs to be done in order to ascertain the exact nature of the cell type, whether MCH is produced locally or has been taken up from the blood and, finally, whether irMCH is associated with other peptide-producing cells, as may be the case in mammals. With this knowledge, some hypotheses may be formed regarding a function for MCH in the fish GI tract and whether it concerns salt and water balance, digestion, or perhaps the modulation of the actions of histamine or other gut hormones.



## CHAPTER 8

### GENERAL DISCUSSION

This thesis has developed from an initial study of MCH ontogeny in amphibians to a more general investigation into the role of MCH as an osmoregulatory hormone in lower vertebrates. During the course of this work, other features of the peptide have been revealed, for example a sexual dimorphism in the response of irMCH neurons to salinity in the amphibian brain, the expression of novel irMCH cell groups linked, perhaps, to reproductive function, and the localization of MCH in peripheral tissues of the trout. This chapter reviews these findings and suggests how future work may elicit more information about MCH and how the peptide may relate to the systems of stress, osmoregulation, reproduction, growth, feeding and immunity.

Studies on MCH gene expression in response to various stimuli in trout and rats have been made possible by the knowledge of the DNA sequences in these species. The primary amino acid sequences of fish and mammalian MCH are sufficiently dissimilar to preclude the construction of a single oligonucleotide probe that might be used to investigate MCH in other animal groups and, given the lack of commercial interest in amphibians, it is also improbable that the amphibian MCH gene will be cloned and characterised in the near future. Immediate investigations into MCH function will probably, therefore, be restricted to fish and mammals.

In the current experiments, the effects of saline challenge were examined in amphibians and trout. In both, a mild osmotic stimulus resulted in increased MCH synthesis. In full strength

seawater, however, trout showed sustained high levels of plasma cortisol and raised osmotic pressures, suggesting that osmoregulatory capability had been lost, and MCH synthesis was significantly depressed. Similarly, in rats, it has been found that drinking a 0.9% salt solution stimulates MCH synthesis (D. Fellmann, personal communication) whilst drinking a 2% salt solution results in a depression of synthesis and release in the majority of MCH neurons in the hypothalamus (Zamir *et al.*, 1986; Nahon *et al.*, 1993; Presse and Nahon). These observations in both fish and rats can be interpreted thus: the stimulatory response of MCH to the more dilute salt solution may result from a mild stress and/or from increased plasma osmolarity. Indeed, it has been suggested that MCH neurons may be osmosensitive since hypothalamic cultures bathed in sucrose solution release more MCH (Fellmann *et al.*, 1993). The stronger salt solution, which may cause hypovolaemia and hypotension, represents a more severe stress, leading to a depression of MCHmRNA. Similar responses of MCH synthesis to other forms of mild or severe stress have been observed previously in fish (Baker and Bird, 1992). In order to expand on these findings, it is necessary to first examine the effects of MCH on the stress axis.

In fish, MCH suppresses the stress-induced release of cortisol by exerting inhibitory effects at hypothalamic and/or pituitary levels (Baker *et al.*, 1986; Green *et al.*, 1991) and in rats icv administration of MCH at the peak of the diurnal rhythm of ACTH release is inhibitory (Bluet-Pajot *et al.*, 1995) although at other times appears to be stimulatory (Jezova *et al.*, 1992). A direct inhibitory effect of MCH on CRH release in rats is precluded by *in vitro* studies (Nahon *et al.*, 1989; Jezova *et al.*, 1992) but an indirect effect, perhaps at the hippocampus or on other afferent pathways to the CRH neurons remains to be investigated.

One apparent contradiction in the current understanding of the relationship between MCH and the HPA axis is in the effects of glucocorticoids on MCH synthesis or release. In mammals glucocorticoids are stimulatory, since dexamethasone restores depleted levels of MCHmRNA following electric footshock or adrenalectomy (Presse *et al.*, 1992). In trout, however, dexamethasone inhibits the stress-induced release of MCH (Green and Baker, 1991) although, since MCH was at its diurnal peak of secretion at the time of examination (1230 h), this may not be a consistent response and should, perhaps, be re-examined throughout the circadian cycle.

To return to the role of MCH in osmotic challenge - it would be informative to repeat the *in-situ* experiments (Chapter 5) but with prior manipulation of some components of the HPI axis in order to try and differentiate between osmotic and stress responses. For example, since in rats it has been demonstrated *in vitro* that CRH depresses MCH synthesis and release (Parkes and Vale, 1992), this influence could be removed by the administration of a CRH inhibitor, such as  $\alpha$ -helical CRH (9-41). The stress-induced rise in plasma cortisol could be abolished by chemical interrenalectomy, using metyrapone, followed by replacement therapy with synthetic steroids. In addition, other hormones, such as atrial natriuretic peptide (ANP) or angiotensin II (AII), which are known to be released during osmotic challenge in euryhaline teleosts (Takei *et al.*, 1985; Westenfelder *et al.*, 1988), could be injected centrally into the trout brain and the response of MCH neurons measured by *in-situ* hybridization.

A further area in which studies of MCH expression would be particularly interesting is that of reproduction. Two groups of irMCH neurons were identified in the amphibian

hypothalamus (Chapter 4). These cells were only stainable in gravid female frogs and were located in the anterior pre-optic nucleus in the telencephalon, regions known to contain amphibian GnRH neurons (Andersen *et al.*, 1992; Sotowska-Brochoka and Licht, 1992). Recently, studies in ovariectomized rats have shown that after 2 d oestrogen replacement, icv administration of MCH into the pre-optic area (POA) results in a surge of luteinizing hormone (LH). Similarly, after 2 d oestrogen replacement, progesterone administration resulted in a pre-ovulatory surge of LH after 4 h, which was abolished by injection of MCH antisera into the POA (Gonzalez *et al.*, 1996b). These results imply that MCH may have a role in the stimulatory control of LH release, via GnRH neurons. It would be of interest, and possibly commercial value, to determine whether a similar relationship between MCH and GnRH exists in fish and whether, as in female amphibians and rats (Knollema *et al.*, 1992), novel irMCH groups become visible in the hypothalamus or telencephalon at particular times in the reproductive cycle, for example before spawning.

Of equal relevance to the fish farming industry is the possible effect of MCH on growth. It has been shown that iv injection of sMCH in rats, results in a delayed but long-lasting elevation of growth hormone (GH) (Kawauchi *et al.*, 1993), although rMCH administered icv showed no effect on either basal or stress-induced plasma GH (Bluet-Pajot *et al.*, 1995). It is as yet anecdotal, but it has been observed in this laboratory that white-reared trout, with higher levels of plasma MCH, tend to be larger than their black-reared siblings, when all other environmental conditions are equal. If this could be verified under controlled experimental conditions, then an investigation into the effects of MCH on GH synthesis or release in fish could prove valuable. Since endogenous MCH levels can be influenced by

manipulation of tank colour, positive effects on growth might be achieved by simple and non-invasive means.

It is equally possible that MCH stimulates appetite and that white-reared fish are larger because they consume more food, but again this is speculative. However, in rats high (5 µg) doses of MCH, injected into the lateral ventricle, double the calorific intake (Qu *et al.*, 1996) whilst lower (1-100 ng) doses have anorectic effects (Presse *et al.*, 1996). Both studies agree, however, that MCHmRNA levels in rat and mouse hypothalami were significantly raised by 24 h fasting. In rats, the majority of irMCH neurons are located in the lateral hypothalamus (LH), a region associated with food intake, whereas irMCH fibres extend to the ventromedial hypothalamus (VMH), a so-called 'satiety centre' (Bittencourt *et al.*, 1992). Lesions of the VMH result in hyperphagia and obesity in adult rodents (Morley, 1987) and increased MCH immunoreactivity and mRNA content in the LH (Deray *et al.*, 1994) suggesting that the VMH exerts inhibitory effects on MCH synthesis.

Investigations on feeding in fish could include the manipulation of peripheral levels of MCH, by changing the tank colour, or central MCH, by immunoneutralization, and observing the effects of these changes on food intake and on the expression of other hormones concerned with feeding, for example CRH, which has anorectic effects when administered icv to rats (reviewed in Morley, 1987). Additionally, the expression of MCHmRNA could be measured by *in-situ* hybridization, following food withdrawal and re-feeding. Again, it may be advantageous to remove the influence of stress-induced cortisol by the means outlined previously.

## Chapter eight

In the periphery, regions of the gastrointestinal tract of fish (current work) and mammals contain irMCH which appears to be expressed in cells of the immune system, in common with other hormones such as arginine vasopressin and ANF (Hervieu *et al.*, 1995; Hervieu and Nahon, 1996). This raises the possibility that MCH may be involved with regulation of immune responses of the gut and hence it would seem appropriate to study the expression of peripheral MCH under conditions of inflammation or infection. As MCH has not been detected in mammalian blood (Takahashi *et al.*, 1995) it is probable that the gut peptide is synthesised *in situ* and has local, paracrine effects. If MCH is within mast cells, a potential autocrine effect on histamine release might prove of importance and the similar distribution of MCH with other hormones might indicate putative functions and provide grounds for comparison between central and peripheral expression.

Thus, an investigation into MCH expression in primary and secondary lymphoid tissues, such as the spleen or thymus, or in immune cell preparations, may be worthwhile. Northern blotting has revealed low levels of a short, possibly deadenylated MCH gene transcript in mouse spleen (Breton *et al.*, 1993a) and in rat, *in-situ* hybridization has shown the presence of scattered irMCH cells of unknown cell type (Hervieu and Nahon, 1995). In neither case could irMCH be visualised by immunocytochemistry in these sites but, given the difficulties of immunostaining gut MCH, experienced in the current work, it is possible that trying a variety of histochemical techniques may produce satisfactory results.

Another interesting discovery in the rat GI tract was that oligonucleotide probes against both sense and anti-sense MCH transcripts revealed similar patterns of hybridization (Hervieu and Nahon, 1995). Furthermore, total RNA from the gut and the hypothalamus

contained both species of MCHmRNA, suggesting that the anti-sense form may play a general role in modulating MCH synthesis. These findings invite further investigation into the sequence and function of anti-sense MCH RNAs, either centrally or peripherally, and it would be interesting to know if such a phenomenon is seen in lower vertebrates or whether it represents a novelty in mammals. When antisense oligomers are administered exogenously, they are generally modulatory rather than activational, that is they exert effects upon systems that have already been stimulated by other factors (reviewed in Morris and Lucion, 1995). It would be informative, therefore, to determine the distribution and activity of anti-sense MCHmRNA in those groups of cells that respond differentially to the same stimulus, for example the trout NLT and LVR neurons under conditions of mild salinity challenge.

A principle hindrance to a greater understanding on how MCH affects the synthesis and release of other hormones and its actions within physiological systems is the lack of knowledge on the distribution of MCH receptors in the brain or periphery. It has been demonstrated, using synthetic MCH analogues, that the ring structure, closed by a disulphide bridge, is essential for biological activity and that the exocyclic arms probably maintain the molecular shape and help receptor binding (Kawazoe *et al.*, 1987; Lebl *et al.*, 1989). Early attempts to iodinate the tyrosine residue (Tyr<sup>13</sup>) abolished bioactivity (Baker *et al.*, 1985b) but by substituting a valine for tyrosine on the c-terminal arm, MCH was monoiodinated whilst retaining bioactivity (Drozd *et al.*, 1995). However, this work is yet to be successfully repeated and has been complicated by the fact that the peptide is very hard to work with, being labile, highly lipophilic and with a tendency to bind non-specifically to laboratory plastic and glassware. These factors have prevented the development of a

standard labelling technique and the distribution of MCH receptors remains unknown. Future work may concentrate on developing alternative MCH analogues or labelling with radioligands other than iodine, for example tritium

The overall conservation of MCH structure between higher and lower vertebrates implies that the peptide has retained functional importance, but whether such functions are necessarily the same in fish and mammals is a moot point. For example, in trout a peripheral antagonism exists between MCH and  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) in the control of colour change, since the peptides have opposing effects on pigment distribution in dermal melanophores (Baker, 1986; Baker, 1988). Clearly, this colour change function does not persist in mammals but a relationship between MCH and  $\alpha$ -MSH remains and can be illustrated by several examples relating to sensory processing and behaviour in rodents. In a few cases, central MCH administration exerts the same effect as the application of endogenous  $\alpha$ -MSH, for example in rats, both peptides are anxiolytic and both stimulate sexual receptivity (Gonzalez *et al.*, 1996a). Other studies have demonstrated a functional antagonism of  $\alpha$ -MSH by MCH, even when the latter is without effect when given alone. For instance, prior icv administration of MCH abolishes  $\alpha$ -MSH-induced aggression (Gonzalez *et al.*, 1996a), grooming, rearing and locomotor activities (Sanchez *et al.*, in press), antagonises the  $\alpha$ -MSH induced enhancement of the hippocampal response to auditory stimuli (Miller *et al.*, 1993) and exerts opposing effects on the extinction of passive avoidance behaviour (McBride *et al.*, 1994).

It is feasible that central antagonism of  $\alpha$ -MSH represents the ancestral role of MCH, from which evolved the peripheral control of teleost chromatic adaptation. Since  $\alpha$ -MSH has



## Chapter eight

other central effects, for example it is a highly potent endogenous anti-pyretic agent which when administered causes dose-related hypothermia in rats (reviewed in DeWied, 1993) and abolishes the interleukin-induced rise of CRH from the hypothalamus (Lyson and McCann, 1993), it may be of value to determine whether opposing effects between  $\alpha$ -MSH and MCH can be demonstrated within the mammalian systems of stress and thermoregulation, and also to explore whether an antagonistic relationship between these peptides exists in lower vertebrates, other than in colour change, returning, in a sense, to the origins of its first discovery.

## APPENDIX

### Hybridization buffer (50ml)

The following are mixed :

25 ml	Formamide (Sigma. Bought as RNAase-free solution and stored at -20°C)
10 ml	20xSSC (below)
2.5 ml	10 mg ml <sup>-1</sup> calf thymus DNA (Sigma. Mixed at 10 mg ml <sup>-1</sup> in DEPC treated water, heated to 80°C to dissolve, then sonicated. Aliquots stored at -20°C and rewarmed before use)
0.5 ml	25 mg ml <sup>-1</sup> yeast tRNA (Boehringer Mannheim. Mixed with DEPC treated water and stored at -20°C)
1 ml	50x Denhardt's (all Sigma. Mix : 1 g ficoll, 1 g polyvinylpyrrolidone, and 1g BSA in 100 ml DEPC water. Stored at -20°C)
10 ml	50% w/v dextran sulphate (Stored at 4°C)
1 ml	Water

Buffer is stored at -20°C and heated to 37°C before use. The final content is : 50% formamide, 4xSSC, 500 mg ml<sup>-1</sup> calf thymus DNA, 200 mg ml<sup>-1</sup> yeast tRNA, 1x Denhardt's, 10% dextran sulphate.

### Sodium saline citrate (20xSSC) stock solution (1 litre)

175.3 g	Sodium chloride (Sigma)
88.2 g	Sodium citrate (Sigma)

Made up to 1 litre with distilled water. Add 1ml diethylpyrocarbonate (DEPC, Sigma) and shake very vigorously for 2 min. Autoclave after 1 hr. For 1xSSC and 2xSSC, dilute with MilliQ water.

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